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Novel species of Botryosphaeriaceae associated with shoot blight of pistachio

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Abstract: Various species of phytopathogenic Botryosphaeriaceae were identified previously from pistachio trees worldwide. Disease symptoms caused by pathogens in Botryosphaeriaceae on pistachio include panicle and shoot blight, leaf defoliation, fruit discoloration and decay. In this study species of Botryosphaeriaceae were collected from blighted pistachio shoots in Arizona, USA, and Greece. The aims of this study were to identify these Botryosphaeriaceae isolates and to test their pathogenicity to pistachio. The fungi were identified based on comparisons of DNA sequence data of the nuclear rDNA internal transcribed spacer region (ITS), a partial translation elongation factor 1-alpha gene (*TEF1*), a partial β-tubulin gene (*TUB2*) and morphological characteristics. Results indicated that some isolates collected from pistachio represent two previously undescribed species, which we described here as *Lasiodiplodia americana* sp. nov. from the United States and *Neofusicoccum hellenicum* sp. nov. from Greece. Field inoculations of *L. americana* and *N. hellenicum* on branches of four pistachio cultivars showed that both *L. americana* and *N. hellenicum* are pathogenic on pistachio. The four pistachio cultivars differed in their susceptibility to the Botryosphaeriaceae species. Results of this study suggested that the two new species of Botryosphaeriaceae need to be monitored carefully to determine the distribution of these pathogens and the possible spread to other areas.

Key words: *Lasiodiplodia*, *Neofusicoccum*, pathogenicity, shoot blight, tree disease

Introduction

The pistachio, *Pistacia vera* L., a member of the cashew family, is a small tree native to the eastern Mediterranean (Cyprus, Turkey to Israel, Syria) and central Asia (Turkmenistan, Uzbekistan, Afghanistan, Tajikistan, Kyrgyzstan) (AL-Saghir and Porter 2012). The edible seeds of *P. vera* have considerable commercial importance worldwide of which the value has increased over the past two decades reaching an annual value of approx $2 billion USA (harvested crop) (AL-Saghir and Porter 2012). Iran (0.472 million metric tons), the United States (0.231 million tons), Turkey (0.15 million tons), China (0.074 million tons), Syria (0.057 million tons) and Greece (0.01 million tons) are considered the main pistachio producers in the world, contributing more than 98% of the world production (http://faostat.fao.org/default.aspx).

Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) is a family of fungi that have a worldwide distribution and cause diseases on a wide range of woody plants (Slippers and Wingfield 2007). Species of Botryosphaeriaceae can cause diseases on buds, fruit panicles, fruits, petioles, rachises, mid rib of leaflets, shoots and branches, resulting in fruit panicles killing, hull and/or kernel decay and dark shell staining, and blight of panicles and shoots on *P. vera* (Michailides and Morgan 1992, 2004). The panicle and shoot blight of pistachio caused by species of Botryosphaeriaceae especially *Neofusicoccum mediterraneum* was considered a great threat to the pistachio industry in California, USA, (Michailides and Morgan 1992, 2004; Chen et al. 2014a) until recently when effective disease management was developed (Morgan et al. 2009).

A number of species of phytopathogenic Botryosphaeriaceae have been reported in regions where *P. vera* trees are planted. *Botryosphaeria dothidea*, *Neofusicoccum parvum* and *N. australe* have been reported from *P. vera* in Australia (Cunnington et al. 2007, Wunderlich et al. 2012, Chen et al. 2014a), *B. dothidea* and *N. parvum* in Greece (Inderbitzin et al. 2010, Chen et al. 2014a), *B. dothidea* and *Diplodia seriata* in South Africa (Swart and Botes 1995, Crous et al. 2000), *N. australe* in Spain (Armengol et al.

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2008) and *Lasiodiplodia theobromae* and *N. mediterraneum* in Arizona, USA, (Inderbitzin et al. 2010, Chen et al. 2014a). In California eight Botryosphaeriaceae species, specifically *B. dothidea*, *Dip. seriata*, *Dothiorella iberica*, *Dot. sarmentorum*, *Lasiodiplodia citricola*, *L. gilanensis*, *N. mediterraneum* and *N. vitifusiforme* (Inderbitzin et al. 2010, Chen et al. 2014a), have been identified from *P. vera*. Pathogenicity test results have indicated *L. citricola* and *L. gilanensis*, followed by *N. mediterraneum*, as the species most pathogenic to the two main cultivars (female Kerman and male Peters) of *P. vera* grown in California (Chen et al. 2014a).

In this study a number of Botryosphaeriaceae isolates were collected from blighted shoots of *P. vera* in USA and Greece, and the isolates exhibited typical morphological characteristics of *Lasiodiplodia* and *Neofusicoccum*. The aims of this study were to describe these species based on molecular data and morphological characteristics and to test the pathogenicity and virulence of the identified species to shoots of different *P. vera* cultivars.

MATERIALS AND METHODS

Isolates.—Diseased samples were collected from pistachio trees at two sites in Arizona, USA, and the regions of Thessaloniki, Chalkidiki and Imathias in Greece. Isolations were made from blighted shoots of pistachio, and isolates showing typical morphology of Botryosphaeriaceae were kept for further identification and characterization. Fungal isolates were isolated from diseased tissues by the method described in Chen et al. (2014a). Fungal isolations were incubated at 25 ± 3 C in Petri dishes containing 2% PDA (Microtech Scientific, Orange, California; 10 g potato dextrose agar, 500 mL water) 2–7 d until colonies were large enough to be examined. To obtain pure single genotype cultures for further research, single hyphal tips from the colonies with typical characteristics of Botryosphaeriaceae were transferred to fresh PDA and incubated at 25 ± 3 C 7–10 d. These isolates are maintained in the culture collection of the Department of Plant Pathology at the University of California at Davis, Kearney Agricultural Research and Extension Center in Parlier, California, USA, and the culture collection of the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), ZhanJiang, GuangDong Province, China. Representative isolates also were deposited with the China Forestry Culture Collection Center (CFCC), Beijing, China. The specimens were deposited in the Collection of Central South Forestry Fungi of China (CSFF), GuangDong.

DNA extraction and PCR amplification.—Single-genotype cultures were grown on 2% PDA for 1 wk at 25 C for DNA extraction. Pure culture mycelia were collected from the medium with a sterile scalpel, and the total genomic DNA was extracted with a FastDNA Kit (BIO 101 Inc., Vista, California). The nuclear rDNA internal transcribed spacer (ITS) region that includes ITS1, the 5.8S rRNA gene and ITS2 were amplified with primers ITS1 and ITS4 (White et al. 1990). Part of the translation elongation factor 1-alpha gene (*TEF1*) was amplified by PCR with primers EF1-728F and EF1-986R (Carbone et al. 1999). The partial β-tubulin gene (*TUB2*) was amplified with primers Bt2A and Bt2B (Glass and Donaldson 1995). The polymerase chain reaction (PCR) of ITS, *TEF1* and *TUB2* were conducted according to Slippers et al. (2004a, 2004b) and Chen et al. (2011). The PCR products were purified with an Ultra Clean PCR Clean-Up Kit (MO BIO Laboratories, Inc., Solana Beach, California).

DNA sequencing and phylogenetic analysis.—The primers used for the PCR reactions were used to sequence the resulting amplicons in both directions. Sequence reactions were run by an automated sequencer by the Division of Biological Sciences sequencing facility, University of California at Davis. Sequences were read and edited with MEGA 4 software (Tamura et al. 2007). Sequences obtained in this study were deposited in GenBank (TABLE I).

Before conducting the phylogenetic analyses for species identification, the ITS, *TEF1* and *TUB2* sequences were subjected to BLAST queries using the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) nucleotide database to obtain preliminary identification. Sequences of the *ex*-type specimen were obtained from GenBank to include in the datasets for phylogenetic analyses (SUPPLEMENTARY TABLE I). Because *TUB2* sequences were unavailable for most of the Botryosphaeriaceae, these sequences were not used for combined analyses. Sequences for each of the three gene regions and the combination of ITS and *TEF1* regions were aligned with the online interface of MAFFT 5.667 (Katoh et al. 2002) with the iterative refinement method (FFT-NS-i settings). Sequence alignments were edited manually in MEGA 4 (Tamura et al. 2007). Sequence alignments for each of the datasets were deposited at TreeBASE (http://treebase.org/treebase-web).

Two phylogenetic analyses were conducted for each of the ITS, *TEF1* and *TUB2* sequences, as well as the ITS and *TEF1* combined datasets. Maximum-parsimony (MP) analyses were performed with PAUP* 4.0b10 and maximum-likelihood (ML) tests were conducted with PhyML 3.0 (Guindon and Gascuel 2003). For MP analyses gaps were treated as a fifth character and the characters were unordered and of equal weight with 1000 random addition replicates. A partition homogeneity test (PHT) was used to determine the congruence of the ITS and *TEF1* datasets (Farris et al. 1995, Huelsenbeck et al. 1996). For analyses of each of ITS, *TEF1*, *TUB2* and combined datasets the most parsimonious trees were obtained with the heuristic search function and tree bisection and reconstruction (TBR) as branch swapping algorithms. MAXTREES were unlimited and branch lengths of zero were collapsed. A bootstrap analysis (50% majority rule, 1000 replicates) was done to determine the confidence levels of the tree-branching points (Felsenstein 1985). Tree length (TL), consistency index (CI), retention index (RI) and the homoplasy index (HI) were used to assess the trees (Hillis and Huelsenbeck 1992).

TABLE I. Isolates used in phylogenetic analyses and pathogenicity tests in this study, and associated data

Species	Isolate No.[a]	Other No.[a]	Host	Location	Collector	GenBank accession No.		
						ITS	*TEF1*	*TUB2*
Lasiodiplodia americana	CERC1960	CFCC50064, 1L91	*Pistachia vera*	Arizona, USA	T.J. Michailides	KP217058	KP217066	KP217074
L. americana	CERC1961[b]	CFCC50065, 2A87	*P. vera*	Arizona, USA	T.J. Michailides	KP217059	KP217067	KP217075
L. americana	CERC1962	CFCC50066, 4E09	*P. vera*	Wilcox, Arizona, USA	T.J. Michailides	KP217060	KP217068	KP217076
Neofusicoccum hellenicum	CERC1947[b]	CFCC50067, 1D05	*P. vera*	Thessaloniki, Greece	T.J. Michailides	KP217053	KP217061	KP217069
N. hellenicum	CERC1948	CFCC50068, 2A94	*P. vera*	Aghios Mamas, Chalkidiki, Greece	T.J. Michailides	KP217054	KP217062	KP217070
N. hellenicum	CERC1953	CFCC50069, 2A28	*P. vera*	Aghios Mamas, Chalkidiki. Greece	T.J. Michailides	KP217055	KP217063	KP217071
N. hellenicum	CERC1957	CFCC50070, 2K90	*P. vera*	Imathias, Greece	T.J. Michailides	KP217056	KP217064	KP217072
N. hellenicum	CERC1959	CFCC50071, 2K95	*P. vera*	Imathias, Greece	T.J. Michailides	KP217057	KP217065	KP217073

[a] CERC, Culture Collection of China Eucalypt Research Centre, Chinese Academy of Forestry, ZhanJiang, GuangDong, China; CFCC, China Forestry Culture Collection Center, Beijing, China.
[b] Isolates are ex-type.

For ML analyses of each of ITS, *TEF1*, *TUB2* and the combined datasets the best models of nucleotide substitution were established with Modeltest 3.7 (Posada and Crandall 1998). The analyses were conducted with PhyML 3.0 (Guindon and Gascuel 2003). Additional ML parameters in PhyML included the retention of the maximum number of 1000 trees and the determination of nodal support by non-parametric bootstrapping with 1000 replicates. The phylogenetic trees were viewed and printed with MEGA 4 (Tamura et al. 2007). For both MP and ML analyses, the sequence of *Guignardia philoprina* (CMW 7063) was used as outgroup taxon (SUPPLEMENTARY TABLE I).

Morphology.—Single hyphal isolates representing each genotype of each species identified by DNA sequence data were transferred to water agar (WA) media (20 g agar, 1 L water: Agar Powder, Beijing Solarbio Science & Technology Co. Ltd.) with sterilized pine needles placed on the agar surface and incubated at 25 C for 2 wk to induce sporulation. Conidia produced from the pycnidia formed on pine needles were mounted in sterilized water on glass slides and examined microscopically. Morphological features of the fungi were determined with a Zeiss Axio Imager.A1 microscope, Zeiss AxioCam MRc digital camera with Zeiss Axio Vision Rel.4.8 software (Carl Zeiss Ltd., Munchen, Germany). Morphological characteristics of conidioma, conidiophore, conidiogenous cell, paraphysis were examined and measured. Measurements were made for these anamorphic structures to determine the smallest and largest. For conidia widths and lengths of 100 were measured for the isolate selected as the holotype and 25 measurements were made for the remaining isolates of each taxon; averaged (mean), standard deviation (std. dev), minimum (min) and maximum (max) measurements presented as (minimum–[average − standard deviation]–[average + standard deviation]–maximum), and their average length: average width ratios (l/w) also were calculated.

Culture characteristics of the Botryosphaeriaceae isolates were based on cultures grown on 2% MEA (20 g malt extract powder, 20 g agar, 1 L water: malt extract powder, Beijing Double-Spiral Microbiological Culture Media Products Factory, Beijing, China) for 3–14 d. To test the growth rates of cultures, representative isolates were selected and grown on 2% MEA for 7 d. A 7 mm plug was removed from these cultures and transferred to the center of 90 mm Petri dishes containing 2% MEA. These cultures were incubated at 5–40 C at five degree intervals. Five replicate plates of each isolate were used at each temperature. The plates were incubated in the dark, and two measurements of colony diameter, at right angles to each other, were taken daily until the fastest growing culture had covered the surface of the plate. The experiment was repeated once. Averages were calculated for each of the eight temperatures. Colony color was determined with the color charts of Rayner (1970).

Pathogenicity tests.—To determine whether the identified species of Botryosphaeriaceae were pathogenic to pistachio trees and to evaluate virulence differences among the identified species, isolates representing different species of

TABLE II. Statistics resulting from phylogenetic analyses

			Maximum parsimony					
Dataset	No. of taxa	No. of bp[a]	PIC[b]	No. of trees	Tree length	CI [c]	RI [d]	HI [e]
ITS	39	525	112	70	201	0.846	0.978	0.154
TEF1	39	317	170	18	376	0.814	0.96	0.186
TUB2	25	422	66	16	236	0.941	0.955	0.059
ITS/*TEF1*	39	842	282	1	595	0.798	0.963	0.202

	Maximum likelihood				
Dataset	Subst. model [f]	NST [g]	Rate matrix	Ti/tv ratio [h]	Rates
ITS	TIMef+G	6	1.0000 4.6409 3.2376 3.2376 12.5245		Gamma
TEF1	HKY+G	2		2.0509	Gamma
TUB2	TrN+G	6	1.0000 2.3880 1.0000 1.0000 7.5672		Gamma
ITS/*TEF1*	TrN+G	6	1.0000 3.2597 1.0000 1.0000 4.7060		Gamma

[a] bp = base pairs.
[b] PIC = number of parsimony informative characters.
[c] CI = consistency index.
[d] RI = retention index.
[e] HI = homoplasy index.
[f] Subst. model, best fit substitution model.
[g] NST = number of substitution rate categories.
[h] Ti/Tv ratio = transition/transversion ratio.

Botryosphaeriaceae were used in the following field inoculation tests. Four pistachio cultivars, Golden Hills, Lost Hills, Kerman and Randy, were selected. Inoculations were conducted with a mycelial PDA plug by the method described in Chen et al. (2014a). In addition to the isolates identified in this study, one isolate of *N. mediterraneum* (2F03, Chen et al. 2014a) was selected as a positive control, and a clean PDA plug served as the negative control for these inoculations. Ten 2 y old branches of each cultivar were inoculated with each of the selected fungal isolates, and 10 additional branches of each cultivars were inoculated with sterile 2% PDA plugs to serve as negative control. The experiment was conducted at the University of California, Kearney Agricultural Research and Extension Center in Parlier, California, USA, in May 2013. Two weeks after inoculation, canker size was measured for each branch. Results of canker or wound length were analysed with EXCEL (2003). Single-factor analysis of variance (ANOVA) was used to define the effects of fungal isolate/control on lesion/wound length. To test the significance among means, F values with $P < 0.05$ were considered significantly different. The standard errors of canker or wound length for each fungal strain and control were calculated.

RESULTS

Isolates.—Four isolates of *Lasiodiplodia* from two sites in Arizona, USA, and 15 isolates of *Neofusicoccum* from Thessaloniki, Chalkidiki and Imathias, Greece, were isolated from pistachio tissues. These Botryosphaeriaceae fungi were isolated from diseased pistachio branches, and each fungal isolate was isolated from different trees. Based on the genotype of ITS, *TEF1* and *TUB2* combined with the characteristics of isolate habitat and origin, three isolates from two sites in Arizona and five isolates from three sites in Greece, which cover all the genotypes, habitat and origin, were selected for further study (TABLE I).

Phylogenetic analysis.—According to the BLAST results using the NCBI nucleotide database, the Botryosphaeriaceae isolates from pistachio in this study can be separated into two groups, the species of *Lasiodiplodia* from USA and *Neofusicoccum* from Greece. Phylogenetic analyses for sequences of isolates in this study (TABLE I) and sequences from GenBank (SUPPLEMENTARY TABLE I) were conducted.

PCR resulted in amplicons of approximately 520 bp for the ITS, 310 bp for the *TEF1* and 420 bp for the *TUB2* gene regions. The partition homogeneity test (PHT) comparing the ITS and *TEF1* gene datasets produced a PHT value of $P = 0.128$, indicating that these two datasets do not have a significant conflict and could be combined in the phylogenetic analyses. The aligned sequences of each dataset of ITS (39 taxa, 525 characters), *TEF1* (39 taxa, 317 characters), *TUB2* (25 taxa, 422 characters) and combination of ITS and *TEF1* (39 taxa, 842 characters) were deposited in TreeBASE (No. S16734). Statistical values for the trees for the maximum parsimony analyses and parameters for the best-fit substitution models of maximum likelihood are provided (TABLE II).

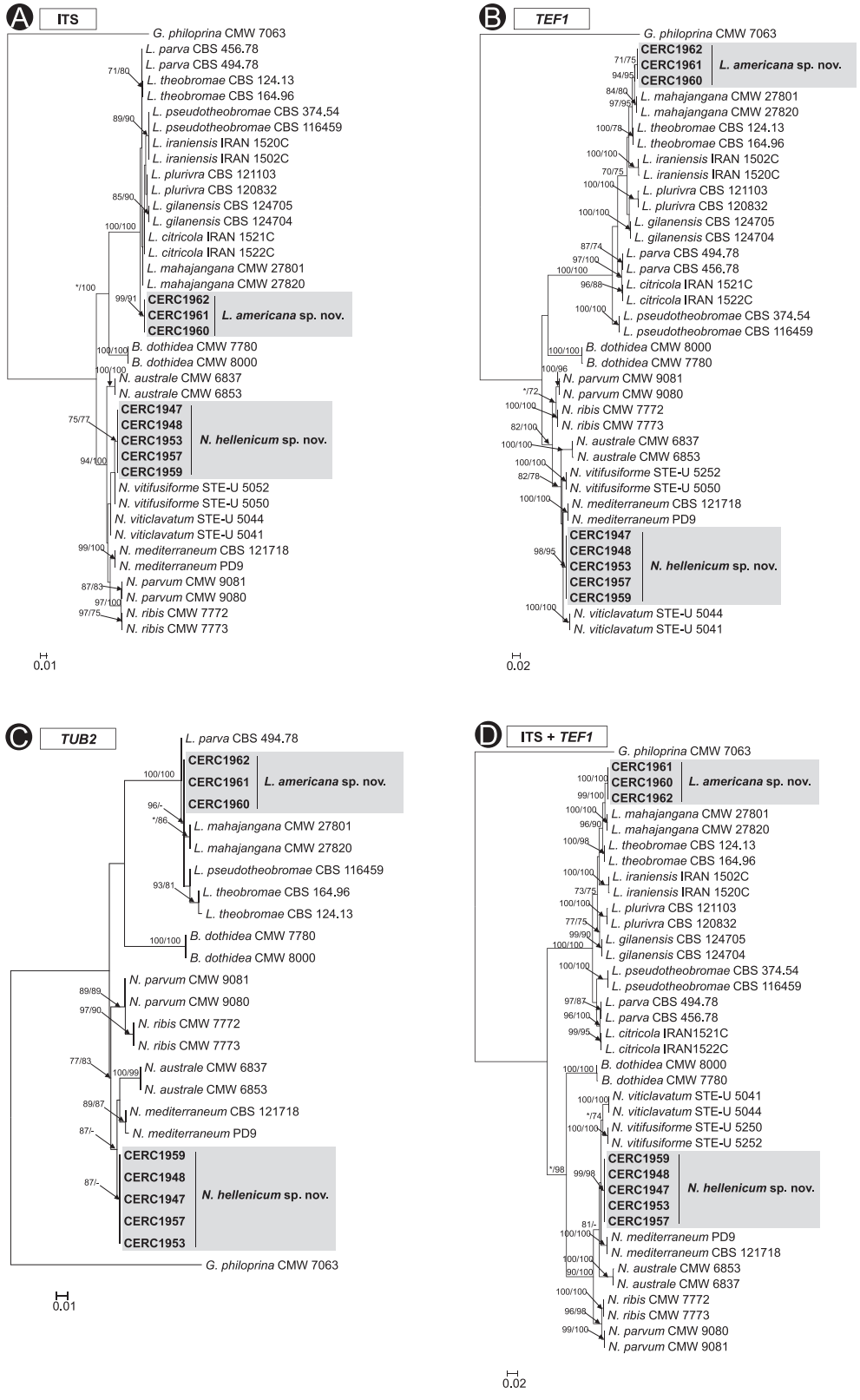

FIG. 1. Phylogenetic tree based on maximum likelihood (ML) analysis for various species in the Botryosphaeriaceae. A. ITS sequences. B. *TEF1* gene sequences. C. *TUB2* gene sequences. D. Combined dataset of ITS and *TEF1* gene sequences. Boldface indicates sequences obtained in this study. Bootstrap values >70% for ML and maximum parsimony (MP) are presented above branches as follows: ML/MP, bootstrap values lower than 70% are marked with asterisk, bootstrap values absent are not shown or marked with minus sign. *Guignardia philoprina* (CMW 7063) represents outgroup.

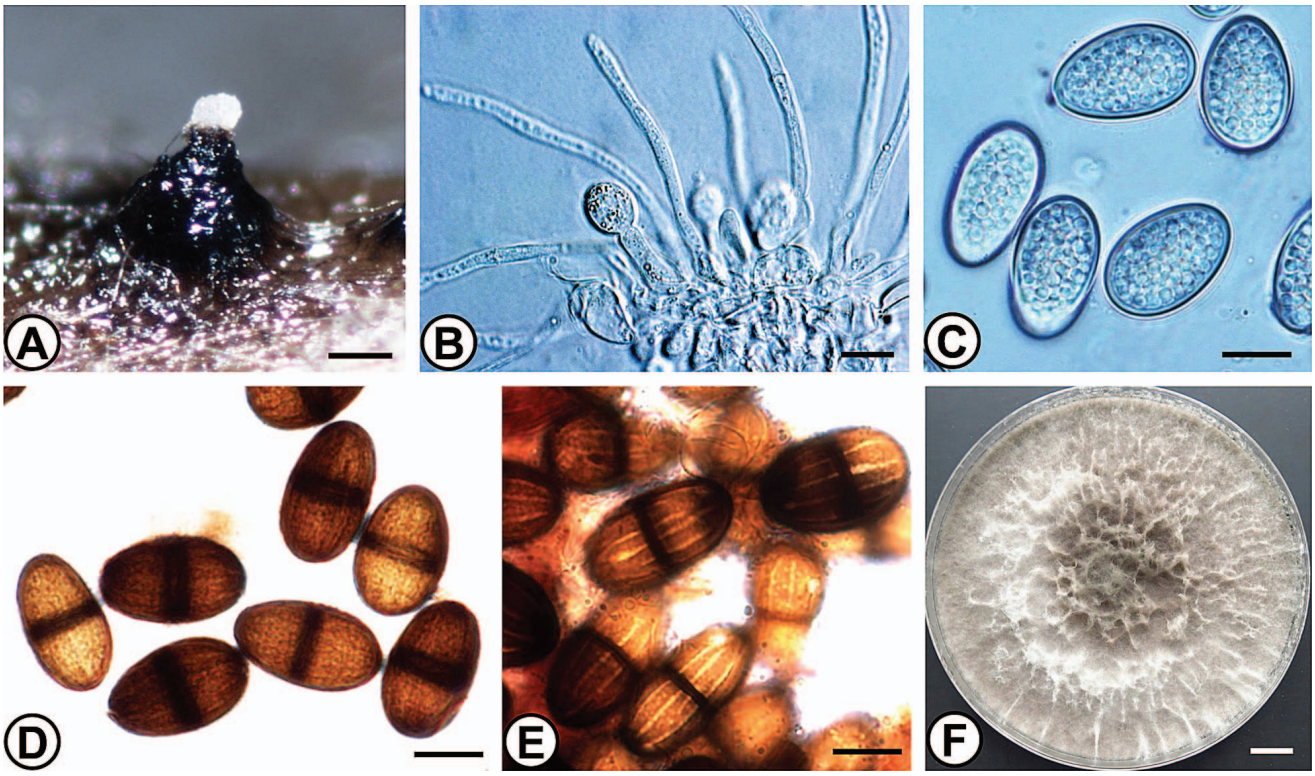

FIG. 2. *Lasiodiplodia americana* holotype. A. Conidiomata on pine needles culture showing oozing of conidia; B. conidia developing on conidiogenous cells between paraphyses; C. hyaline immature conidia; D, E. mature conidia in two different focal planes to show septum (D) and longitudinal striations (E); F. living culture after 7 d on MEA (front). Bars: A = 100 μm; B = 20 μm; C, D, E = 10 μm; F = 1 cm.

Based on the phylogenetic analyses of ITS, *TEF1*, *TUB2* and combination of ITS and *TEF1* sequences by ML and MP, isolates CERC1960, CERC1961 and CERC1962 from pistachio in USA grouped into the clade of *Lasiodiplodia*. These isolates were found to be distinct from other known phylogenetically related species in the genus by congruent distinction in all the four datasets and supported by high bootstrap value or formed an independent clade (ML/MP: ITS = 99%/91%, *TEF1* = 71%/75%, *TUB2*- bootstrap value not available but formed one distinguished clade, combination of ITS and *TEF1*, 100%/100%) (FIG. 1). The isolates, CERC1947, CERC1948, CERC1953, CERC1957 and CERC1959, from pistachio in Greece grouped into the genus of *Neofusicoccum* and were found to be distinct from the known species in the genus by congruent distinction in all three datasets and supported by high bootstrap value (ML/MP: ITS = 75%/77%, *TEF1* = 98%/95%, *TUB2* = 87%/−, combination of ITS and *TEF1*, 99%/98%) (FIG. 1). The phylogenetic analyses indicated that the isolates from USA and isolates from Greece represent two distinct undescribed species.

Morphology and taxonomy.—The eight isolates of the Botryosphaeriaceae from pistachio in USA and Greece produced anamorph fruiting structures on pine needles on WA media within 2–3 wk. Teleomorph structures were not observed. All isolates were separated into two main groups based on conidial morphology. Three isolates from USA produced dark, septate and striate conidia typical for *Lasiodiplodia* spp. (FIG. 2), and five isolates from Greece produced hyaline, *Neofusicoccum*-like conidia (FIG. 3). Based on anamorph morphology and DNA sequence comparisons, we described two new species.

TAXONOMY

Lasiodiplodia americana S.F. Chen, G.Q. Li & T.J. Michailides, sp. nov. FIG. 2

MycoBank MB810934

Typification: UNITED STATES. Arizona: from twigs of one *Pistacia vera* cultivar Kerman, 5 Oct 2007, *T.J. Michailides*. A sample of pine needles on surface of WA media inoculated with isolate *CERC1961* was dried and deposited as Herb. *CSFF2008* (HOLOTYPE). Culture EX-TYPE *CERC1961* = *CFCC50065*.

Etymology: After the United States of America where it was first found.

Conidiomata pycnidial, produced on pine needles on WA within 2–4 wk, solitary, globose to ovoid, dark

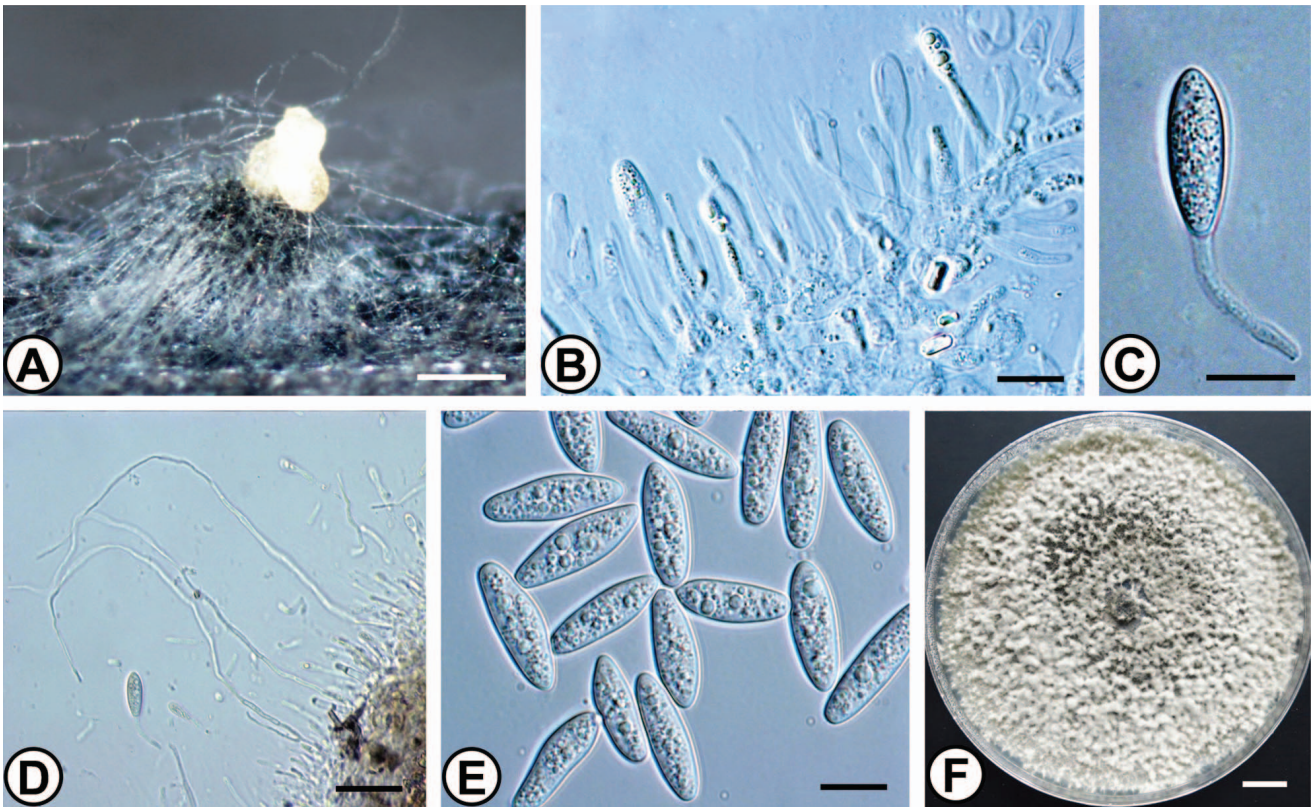

FIG. 3. *Neofusicoccum hellenicum* holotype. A. Conidiomata on pine needles culture showing oozing of conidia; B. immature, developing conidia produced by conidiogenous cells; C. conidiogenous cells and developing conidia; D. long paraphyses; E. mature conidia with granular contents; F. living culture after growing ten days on MEA (front). Bars: A = 100 µm; B = 20 µm; C, D, E = 10 µm; F = 1 cm.

brown to black, up to 446 µm wide (av. 50 conidiomata 180 µm), up to 536 µm high (av. 50 conidiomata 196 µm), embedded in needle tissue, semi-immersed to superficial, unilocular, with a central ostiole, exuding conidia in a white mucoid mass (FIG. 2A); wall 3–6 cell layers thick, outer layers composed of dark brown textura angularis, becoming thin-walled and hyaline toward the inner region. Conidiophores reduced to conidiogenous cells (FIG. 2B). Conidiogenous cells holoblastic, discrete, hyaline, cylindrical, proliferating percurrently to form one or two annellations or proliferating at the same level giving rise to periclinal thickenings, 10–18 × 3–5 µm (av. 50 Conidiogenous cells 13 × 3.5 µm) (FIG. 2B). Paraphyses hyaline, cylindrical, 1–3-septate, apical cell with rounded tip, sometimes branched, up to 90 µm long, 2–3.5 µm wide (FIG. 2B). Conidia initially aseptate, hyaline, ellipsoidal to ovoid, rounded at apex, base mostly truncate, with granular content (FIG. 2C), becoming dark brown and one-septate after discharge from the pycnidia, with melanin deposits on the inner surface of the wall arranged longitudinally giving a striate appearance to the conidia (FIGS. 2D, E), (14.0–)17.5–20.5(–24.5) × (10.5–)11.5–13.0(–15.0) µm (av. 200 conidia 19.3 × 12.3 µm, l/w = 1.57) (TABLE III, FIG. 2E).

Culture characters: On MEA *Lasiodiplodia americana* fluffy with an uneven margin, growing fast, with abundant aerial mycelia reaching to the lid of Petri plate (FIG. 2F). Colony aerial mycelia white when young, turning pale smoke gray (21′′′′f) to deep grayish olive (21′′′′i) at the surface and deep slate green (33′′′′k) to dark slate blue (39′′′′k) on the reverse after 7 d in the dark at 30 C. Optimal growth 30(–35) C, covering the 90 mm plates after 2 d, no growth at 5 C. After 48 h colonies at 10 C, 15 C, 20 C, 25 C, 30 C, 35 C and 40 C reached 9 mm, 18 mm, 40 mm, 65 mm, 85 mm, 83 mm and 9 mm, respectively.

Teleomorph: Not observed.

Habitat: Branches and twigs of *Pistacia vera* cultivar Kerman.

Hosts and distributions: Pistacia vera, in Arizona, USA.

Specimens examined: UNITED STATES. ARIZONA: from twigs of one *Pistacia vera* cultivar Kerman, 25 Sep 2007, *T. J. Michailides*. A sample of pine needles on surface of WA media inoculated with isolate *CERC1960* was dried and

TABLE III. Conidia sizes, conidia colors and culture growth rates of *L. americana* and *N. hellenicum*

Species	Isolate No.	(l × w) conidial size (ìm)[a]	(l × w) mean ± SD (μm)[b]	l/w[c]	Isolate color	Colony diam (mm)[d]							
						5 C	10 C	15 C	20 C	25 C	30 C	35 C	40 C
L. americana	CERC1960	(16.0–)19.1–21.8(–24.2) × (10.7–)12.5–13.3(–14.9)	19.9.±0.9 × 12.9±0.4	1.54	Hyaline to dark brown	7	10	23	45	65	**88**	**88**	9
L. americana	CERC1961	(15.2–)17.6–20.6(–23.6) × (10.8–)11.4–13.0(–14.4)	19.1±1.5 × 12.2±0.8	1.57	Hyaline to dark brown	7	8	16	38	60	**80**	**80**	8
L. americana	CERC1962	(14.0–)17.5–20.3(–22.4) × (9.8–)11.3–12.5(–14.5)	18.9±1.4 × 11.9±0.6	1.59	Hyaline to dark brown	7	9	15	37	70	**87**	82	10
L. americana	Average	(14.0–)17.8–20.8(–24.2) × (10.7–)11.6–13.0(–14.9)	19.3±1.5 × 12.3±0.7	1.57		7	9	18	40	65	**85**	83	9
N. hellenicum	CERC1947	(18.4–)20.6–23.4(–25.9) × (5.4–)6.2–6.8(–7.9)	22.0±1.4 × 6.5±0.3	3.4	Hyaline	7	11	23	61	86	**90**	10	7
N. hellenicum	CERC1948	(18.5–)21.2–25.0(–25.4) × (5.9–)7.1–7.9(–9.0)	23.1±1.9 × 7.5±0.4	3.1	Hyaline	7	10	22	63	**89**	88	9	7
N. hellenicum	CERC1959	(21.0–)22.2–26.6(–27.5) × (6.0–)6.4–7.8(–9.2)	24.4±2.2 × 7.1±0.7	3.4	Hyaline	7	11	20	59	**90**	86	17	7
N. hellenicum	Average	(18.4–)21.4–24.8(–26.5) × (5.4–)6.6–7.5(–9.0)	23.1±1.7 × 7.1±0.5	3.3		7	11	22	61	**88**	**88**	12	7

[a] l × w, length × width, minimum–(average−standard deviation)–(average+standard deviation)–maximum.

[b] l × w, length × width; SD, standard deviation.

[c] l/w, average length/average width.

[d] For each culture, a 7.0 mm culture plug was transferred to the center of a 90-mm Petri dish containing MEA, and incubated at the temperature indicated. Measurements of colony diam were taken after 48 h for isolates of *L. americana*, and 120 h for isolates of *N. hellenicum*. Measurements in boldface represent optimal growth temperatures.

deposited as Herb. *CSFF2007*. Culture *CERC1960 = CFCC50064*; ARIZONA: from twigs of one *Pistacia vera* cultivar Kerman, date unknown, *T.J. Michailides*. A sample of pine needles on surface of WA media inoculated with isolate *CERC1962* was dried and deposited as Herb. *CSFF2009*, culture *CERC1962 = CFCC50066*.

Notes:. *Lasiodiplodia americana* phylogenetically is closely related to *L. mahajangana*, as well as *L. theobromae* and *L. iraniensis*, while it can be distinguished from these species by the size and shape of their conidia. Conidia of *L. americana* (19.3 × 12.3 μm; l/w = 1.6) are longer and wider than *L. mahajangana* (av. 17.5 × 11.5; l/w = 1.4) (Begoude et al. 2010) but shorter and narrower than those of *L. theobromae* (av. 26.2 × 14.2; l/w = 1.9) (Alves et al. 2008) and *L. iraniensis* (av. 20.7 × 13; l/w = 1.6) (Abdollahzadeh et al. 2010). Optimum growth temperature *of L. americana* (30–35 C) is different from *L. mahajangana* (25–30 C) (Begoude et al. 2010) and *L. iraniensis* (25–30 C) (Alves et al. 2008).

Neofusicoccum hellenicum S.F. Chen, G.Q. Li & T.J. Michailides, sp. nov. FIG. 3

MycoBank MB810935

Typification: GREECE. THESSALONIKI: from twigs of one *Pistacia vera* cultivar Aegina, 23 Oct 1997, *T.J. Michailides*. A sample of pine needles on surface of WA media inoculated with isolate *CERC1947* was dried and deposited as Herb. *CSFF2010* (HOLOTYPE). Culture EX-TYPE *CERC1947 = CFCC50067*.

Etymology: Named after ''Hellas'' for Greece where it was first found.

Conidiomata pycnidial, produced on pine needles on WA within 2–4 wk, solitary, globose, dark brown to black, up to 439 μm wide (av. 50 conidiomata 148 μm), up to 469 μm high (av. 50 conidiomata 197 μm), embedded in needle tissue, semi-immersed to superficial, unilocular, with a central ostiole, exuding conidia in a white mucoid mass (FIG. 3A); wall 3–7 cell layers thick, outer layers composed of dark brown textura angularis, becoming thin-walled and hyaline toward the inner region. Conidiophores lining the inner layer of the conidioma, hyaline, smooth, 0–1-septate, 7–20 × 2.5–4 μm (FIG. 3B). Conidiogenous cells integrated, phialidic, subcylindrical, 8.5–16.5 × 1.5–3.5 μm (av. 50 conidiogenous cells 12.9 × 2.6 μm), proliferating several times percurrently near apex, rarely with minute periclinal thickening (FIG. 3 C). Paraphyses hyaline, cylindrical, 2–8-septate, sometimes branched, up to 160 μm long, 1.5 × 3.5 μm wide (FIG. 3D). Conidia hyaline, smooth, thin-walled, fusoid-ellipsoidal, widest in the middle or in the upper third, apex subobtuse, base subtruncate, with granular content, somewhat flattened with minute marginal frill, (18.5–)21.5–25.0(–27.5) × (5.5–)6.5–7.5 (–9.0) μm (av. 200 conidia 23.1 × 7.1 μm; l/w = 3.3) (TABLE III, FIG. 3E).

Culture characters: On MEA *Neofusicoccum hellenicum* fluffy with uneven margin, growing moderate, colonies were moderately dense, cottony aerial mycelium toward the edge of colony (FIG. 3F). Colony mycelia initially white turning apricot orange (11′) from the middle of colonies within 3 d, becoming deep mouse gray (15′′′′′i) to blackish mouse gray (15′′′′′m) at the surface and dark olive gray (23′′′′′i) to iron gray (23′′′′′k) on the reverse after 7 d in the dark at 25 C. Optimal growth temperature 25–30 C, covering 90 mm plates after 5 d, no growth at 5 C or 40 C. After 120 h colonies at 10 C, 15 C, 20 C, 25 C, 30 C and 35 C reached 11 mm, 22 mm, 61 mm, 88 mm, 88 mm and 12 mm, respectively.

Teleomorph: Not observed.

Habitat: Branches and twigs of *Pistacia vera* cultivar Aegina.

Hosts and distribution: Pistacia vera, in Greece.

Specimens examined: GREECE. AGHIOS MAMAS: Chalkidiki, from twigs of one *Pistacia vera* cultivar Aegina, 3 Jul 2008, *T.J. Michailides*. A sample of pine needles on surface of WA media inoculated with isolate *CERC1948* was dried and deposited as Herb. *CSFF2011*. Culture *CERC1948 = CFCC50068*; GREECE. IMATHIAS: from twigs of one *Pistacia vera* cultivar Aegina, 6 Jun 2005, *T.J. Michailides*. A sample of pine needles on surface of WA media inoculated with isolate *CERC1959* was dried and deposited as Herb. *CSFF2012*. Culture *CERC1959 = CFCC50071*.

Notes: Neofusicoccum hellenicum is phylogenetically closer to *N. mediterraneum*, *N. vitifusiforme* and *N. viticlavatum*, while it can be distinguished from these species by conidia size. Conidia of *N. hellenicum* (23.1 × 7.1 μm; l/w = 3.3) are shorter and wider than that of *N. mediterraneum* (av. 24 × 6; l/w = 4) (Crous et al. 2007) but longer than *N. vitifusiforme* (av. 20 × 6; l/w = 3.3) and *N. viticlavatum* (av. 17 × 7; l/w = 2.4) (van Niekerk et al. 2004).

Pathogenicity tests.—Two isolates of *L. americana* (CERC1960, CERC1961), two isolates of *N. hellenicum* (CERC1947, CERC1948) and one isolate of *N. mediterraneum* (2F03) (Chen et al. 2014a) were selected for pistachio branch inoculations. All tested isolates produced significant longer lesions than the negative control on attached branches in 2 wk (FIG. 4). Overall *L. americana* was more pathogenic than *N. hellenicum* while lesion lengths produced by *N. hellenicum* and *N. mediterraneum* were similar. The cultivars Lost Hills and Kerman were more susceptible than Golden Hills and Randy (FIG. 4). Mycelial plugs of *L. americana* were able to kill the branches of

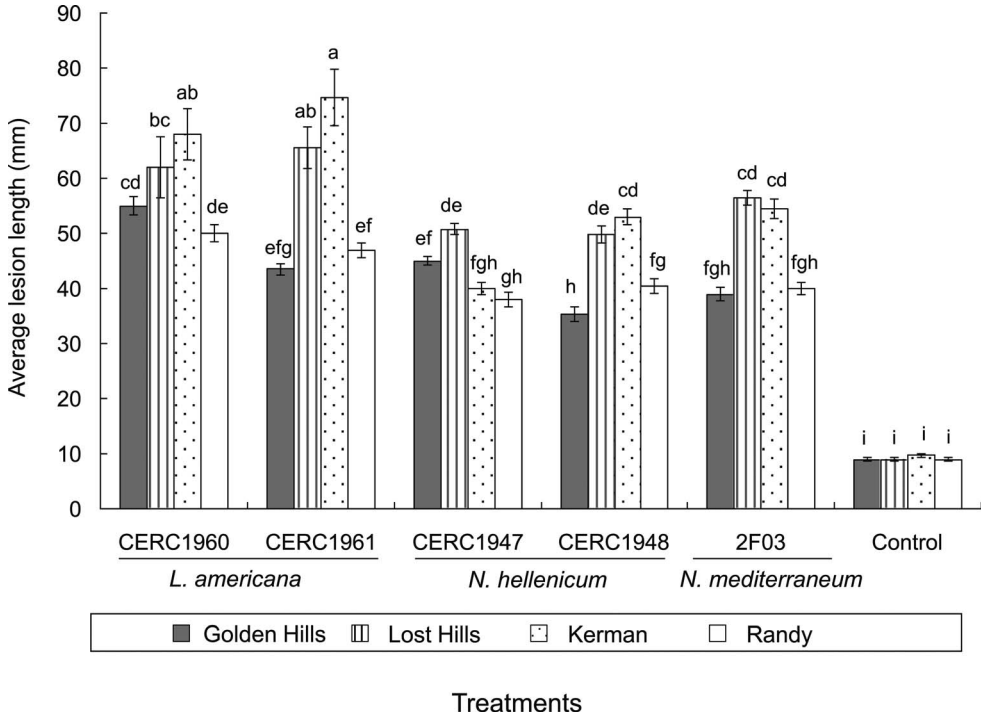

FIG. 4. Bar graph showing the average lesion length (mm) resulting from inoculation trials with *L. americana*, *N. hellenicum* and *N. mediterraneum* onto branches of four pistachio cultivars. Vertical bars represent standard error of means. Letters above bars indicate treatments that were significantly different ($P = 0.05$).

pistachio cultivars Lost Hills and Kerman within 2 wk (SUPPLEMENTARY FIG. 1).

DISCUSSION

In this study two undescribed species of Botryosphaeriaceae were identified and described from diseased pistachio branches. These include *Lasiodiplodia americana* from USA and *Neofusicoccum hellenicum* from Greece. The identification of these fungi was supported by DNA sequence comparisons as well as by morphological characteristics. Pathogenicity tests showed that the two species are capable of causing lesions on pistachio branches.

DNA sequence comparisons of multiple gene regions combined with morphological characteristics have been used in species identification of Botryosphaeriaceae (Abdollahzadeh et al. 2010, Liu et al. 2012, Marques et al. 2013, Machado et al. 2014, Netto et al. 2014). In this study the two new species, *L. americana* and *N. hellenicum*, clearly could be distinguished from other closely related species by DNA sequence comparisons of ITS, *TEF1* and *TUB2*, as well as by the combined dataset of ITS and *TEF1* sequences. The species, *L. americana* and *N. hellenicum*, also can be distinguished from other phylogenetically related species based on morphology, especially by the characteristics of the conidia and to a lesser extent on the presence or absence and the characteristics of paraphyses in the conidiomata (van Niekerk et al. 2004, Crous et al. 2007, Damm et al. 2007, Alves et al. 2008, Abdollahzadeh et al. 2010, Begoude et al. 2010, Machado et al. 2014, Netto et al. 2014).

Lasiodiplodia americana is identified as a new species in the genus *Lasiodiplodia* whereby phylogenetic analyses indicated that *L. americana* formed a separated clade. Although *L. americana* is phylogenetically closely related to *L. mahajangana* and *L. theobromae,* morphological comparisons showed that conidia of *L. americana* are larger than *L. mahajangana* (Begoude et al. 2010) but smaller than *L. theobromae* (Alves et al. 2008).

The newly described species *N. hellenicum* is phylogenetically most closely related to *N. mediterraneum*, whose type specimens were described from a collection from a Greek island (Crous et al. 2007). However, six nucleotides in the ITS region, eight nucleotides in the *TEF1* region and five in the *TUB2* region distinguish *N. hellenicum* from *N. mediterraneum*. Considering the phylogenetic data, the isolates of *N. hellenicum* formed a clade strongly supported in the ML and MP analyses. *N. hellenicum* also can be distinguished from *N. mediterraneum* based on conidial size, which is wider than that described for *N. mediterraneum* (Crous et al. 2007).

The pathogenicity test indicated that both *L. americana* and *N. hellenicum* are pathogenic to branches of all the tested pistachio cultivars. Inoculation results in this study showed that *L. americana* produced significantly longer lesions than the negative control in relatively short time. *Lasiodiplodia americana* and two previously identified species of *Lasiodiplodia* from pistachio in California were able to kill the branches of some cultivars within 2 wk (Chen et al. 2014a). Moreover, Chen et al (2014a) showed that *N. mediterraneum* is distributed widely on pistachio in California and now is considered the main causal agent of pistachio panicle and shoot blight in California. Results in the current study indicated that *N. hellenicum* also is pathogenic to the tested cultivars and it is similar in pathogenicity to *N. mediterraneum* but less pathogenic than *L. americana*. Although the precise distribution of *N. hellenicum* and *N. mediterraneum* is not known in pistachios grown in Greece, panicle and shoot blight (described initially as Camarosporium blight in Greece [Zachos et al. 1974]) is considered one of the most devastating diseases of Greek pistachios (T.J. Michailides, unpubl data). Therefore *N. hellenicum* and *N. mediterraneum* might play an important role in causing this disease on pistachio in Greece.

In USA pistachio is planted mainly in California, although smaller acreage is growing in Arizona and New Mexico. Approximately 114 000 ha (285 000 acres) of pistachio planted now in California playing a major role in the economy of the state. In Greece, panicle and shoot blight is considered a serious threat of pistachio trees in almost all pistachio-growing areas (T.J. Michailides unpubl data). Recently a relatively great number of fungi in the Botryosphaeriaceae were reported not only from pistachios in USA and Greece (Chen et al. 2014a) but also from other nut crops such as almond (*Prunus dulcis*) in California (Inderbitzin et al. 2010, Chen et al. 2013b) and Spain (Gramaje et al. 2012), olive in Spain and California (Moral et al. 2010) and walnut (*Juglans regia*) in California (Chen et al. 2013a, 2014b). Because *L. americana* and *N. hellenicum* isolates identified in this study were able to cause disease on pistachio trees, these pathogens need to be monitored carefully in Arizona and Greece, respectively, in the event they spread and cause serious pistachio diseases not only in Arizona and Greece but also in other regions and on other tree crops.

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