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Fusarium euwallaceae sp. nov.—a symbiotic fungus of *Euwallacea* sp., an invasive ambrosia beetle in Israel and California

S. Freeman[1]
M. Sharon
M. Maymon
Department of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan 50250, Israel

Z. Mendel
A. Protasov
Department of Entomology, ARO, The Volcani Center, Bet Dagan 50250, Israel

T. Aoki
Genetic Resources Center, National Institute of Agrobiological Sciences, 2-1-2, Kannondai, Tsukuba, Ibaraki 305-8602 Japan

A. Eskalen
Department of Plant Pathology and Microbiology, University of California, Riverside, California 92521

K. O'Donnell
Bacterial Foodborne Pathogens and Mycology Research Unit, NCAUR-ARS-USDA, 1815 N. University Street, Peoria, Illinois 61604

Abstract: The invasive Asian ambrosia beetle *Euwallacea* sp. (Coleoptera, Scolytinae, Xyleborini) and a novel *Fusarium* sp. that it farms in its galleries as a source of nutrition causes serious damage to more than 20 species of live trees and pose a serious threat to avocado production (*Persea americana*) in Israel and California. Adult female beetles are equipped with mandibular mycangia in which its fungal symbiont is transported within and from the natal galleries. Damage caused to the xylem is associated with disease symptoms that include sugar or gum exudates, dieback, wilt and ultimately host tree mortality. In 2012 the beetle was recorded on more than 200 and 20 different urban landscape species in southern California and Israel respectively. *Euwallacea* sp. and its symbiont are closely related to the tea shot-hole borer (*E. fornicatus*) and its obligate symbiont, *F. ambrosium* occurring in Sri Lanka and India. To distinguish these beetles, hereafter the unnamed xyleborine in Israel and California will be referred to as *Euwallacea* sp. IS/CA. Both fusaria exhibit distinctive ecologies and produce clavate macroconidia, which we think might represent an adaption to the species-specific beetle partner. Both fusaria comprise a genealogically exclusive lineage within Clade 3 of the *Fusarium solani* species complex (FSSC) that can be differentiated with arbitrarily primed PCR. Currently these fusaria can be distinguished only phenotypically by the abundant production of blue to brownish macroconidia in the symbiont of *Euwallacea* sp. IS/CA and their rarity or absence in *F. ambrosium*. We speculate that obligate symbiosis of *Euwallacea* and *Fusarium*, might have driven ecological speciation in these mutualists. Thus, the purpose of this paper is to describe and illustrate the novel, economically destructive avocado pathogen as *Fusarium euwallaceae* sp. nov. S. Freeman et al.

Key words: Ambrosia beetle, ap-PCR, *EF-1α*, *Euwallacea fornicatus*, *Fusarium ambrosium*, *Fusarium solani* species complex, gene genealogies, molecular phylogenetics, morphology, mutualism, mycangia, *Persea americana*, *RPB1*, *RPB2*

Introduction

The avocado industry in Israel and California is threatened by the invasive ambrosia beetle *Euwallacea* sp. IS/CA (Coleoptera, Xyleborini) from Asia and the novel ambrosia *Fusarium* sp. that it cultivates in its galleries (Fig. 1A–F). Analyses of multilocus DNA sequence data (Mendel et al. 2012, Kasson et al. 2013) and laboratory-rearing experiments (Freeman et al. 2012b) suggest the fungal symbiont represents an independent evolutionary lineage. The exotic xyleborine (Hulcr et al. 2007) initially was reported incorrectly as the tea shot-hole borer (TSHB) *E. fornicatus* (Eichhoff) (Eskalen et al. 2012, Mendel et al. 2012), but based on subsequent molecular phylogenetic analyses it was renamed, albeit prematurely, the polyphagous shot-hole borer (PSHB, Rugman-Jones and Stouthamer 2012).

The spectacular adaptive radiation of ~ 1400 Xyliborini over the past 20 000 000 y is attributed to their ability to successfully colonize large numbers of host trees species and their highly inbred haplodiploid sex-determination system in which sib mating is obligate (Jordal et al. 2000, Farrell et al. 2001). Among the Xyleborini, in contrast to males that are haploid, flightless, lack mycangia and never stray far from the natal gallery host tree, adult females are diploid (Fig. 1F) and vector fungal symbionts in

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[1]Corresponding author. Dept. of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan 50250, Israel. E-mail: freeman@volcani.agri.gov.il

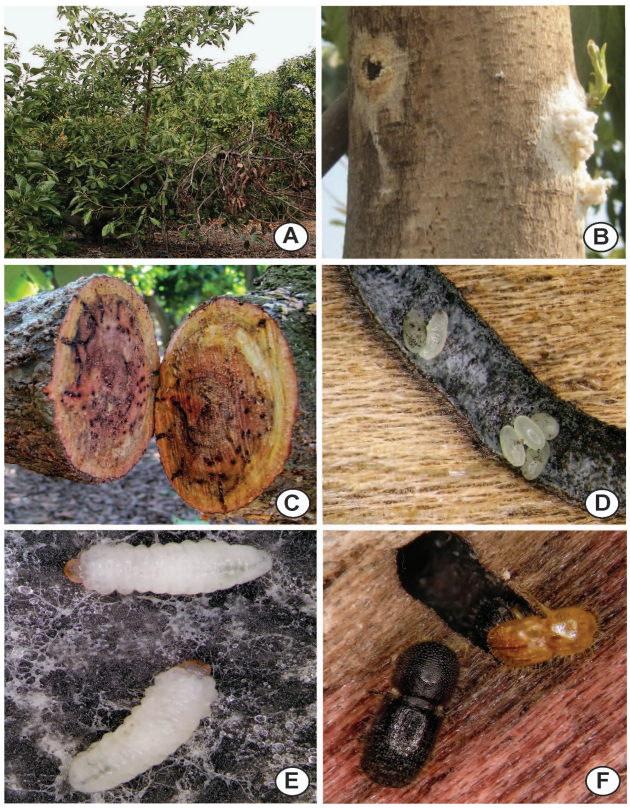


FIG. 1. Symptomatic avocado infested with *Euwallacea* sp. IS/CA (A–B), galleries (C), and beetle life stages (D–F). A. Avocado dieback and wilt. B. Mannoheptulose exudates surrounding gallery entrance. C. Transverse section of tree with extensive galleries. D. Close up of gallery showing eggs and wall covered with *Fusarium euwallaceae*. E. Larvae feeding on *F. euwallaceae* on PDA supplemented with finely ground sawdust of avocado wood (Freeman et al. 2012b). F. Adult female (left) and male (right) beetles.

mycangia from the gallery after they mate with a male sib (Francke-Grosmann 1967, Six 2003). Also, in contrast to bark beetles that reproduce in the phloem (Six 2012), ambrosia beetles construct galleries in the xylem (FIG. 1D). The spread of their fungal symbiont can obstruct water and mineral transport, resulting in dieback, wilt and mortality of the host tree. *Euwallacea* sp. IS/CA through its fungal symbiont poses a major threat to avocado production (*Persea americana* Miller) in Israel and California (Eskalen et al. 2012, Freeman et al. 2012a, Mendel et al. 2012). In addition to avocado, the beetle can complete its life cycle on at least 20 other live trees, including box elder (*Acer negundo* L.), castor bean (*Ricinis communis* L.), several oaks (*Quercus* spp.) and California sycamore (*Platanus racemosa* Nutt.). Surveys conducted in 2012 in Los Angeles, Orange and San Bernardino counties, for example, revealed that the beetle had attacked more than 200 different healthy tree species, resulting in serious damage to more than half of them (Eskalen et al. 2013). Ambrosia beetles typically attack dead or dying hosts; therefore the atypical colonization of diverse healthy hosts by *Euwallacea* sp. IS/CA and several other invasive ambrosia beetles (e.g. Koch and Smith 2008) is attributed to an olfactory mismatch with the foreign hosts that it encounters outside its native habitat (Hulcr and Dunn 2011).

The independent completion of Koch's postulates (Eskalen et al. 2012, Mendel et al. 2012) established that the *Fusarium* sp., which is cultivated in galleries by adult female *Euwallacea* sp. IS/CA for nutrition of the larvae and adults (FIG. 1D), was responsible for the wilt and dieback on avocado in Israel and California. Results of rearing experiments also suggest that the beetle-fungus mutualism is obligate (FIG. 1E; Freeman et al. 2012b), given that the larvae can complete their life cycle on a culture of this fungus but not on that of *F. ambrosium* (Gadd & Loos) Agnihothrude & Nirenberg, the symbiont of the TSHB, *E. fornicatus* (Gadd and Loos 1947, Brayford 1987). Therefore, the common names PSHB and TSHB have been misapplied to *Euwallacea* sp. IS/CA. The fusaria symbionts of both beetles produce clavate, multiseptate macroconidia that may represent an adaptation for the symbiosis. Because these differ from the hallmark fusiform macroconidium produced by most fusaria, the initial collection of *F. ambrosium* from galleries in Chinese tea (*Camellia sinensis* [L.] Kuntze) in Sri Lanka (as Ceylon) was described as *Monacrosporium ambrosium* Gadd & Loos (Gadd and Loos 1947). Apparently unaware of the Gadd and Loos (1947) study, Brayford (1987) redescribed this species four decades later as *F. bugnicourtii*. Nirenberg (1990) subsequently recombined the species in *Fusarium* as *F. ambrosium*. Molecular phylogenetic analyses of multilocus DNA sequence data (Mendel et al. 2012, Kasson et al. 2013) indicate that the symbionts of *E. fornicatus* and *Euwallacea* sp. IS/CA comprise a novel subclade within Clade 3 of the *Fusarium solani* species complex (FSSC, O'Donnell et al. 2008).

Here we report that the *Fusarium* symbiont of *Euwallacea* sp. IS/CA and *F. ambrosium* were resolved by multilocus molecular phylogenetics as independent evolutionary lineages that exhibit distinctive ecologies. Laboratory-rearing experiments (Freeman et al. 2012b) indicate that obligate symbiosis of *Euwallacea-Fusarium* might have driven ecological speciation of these mutualists. Our phenotypic analyses suggest that the symbiont of *Euwallacea* sp. IS/CA might be distinguished from *F. ambrosium* by the abundant production of blue to brownish macroconidia in the former and their rarity or absence in the latter. Thus, the purpose of this paper is to describe and illustrate the novel, economically

destructive avocado pathogen as *Fusarium euwallaceae* sp. nov.

MATERIALS AND METHODS

Fungal isolates and growth conditions.—The *Fusarium* spp. strains (TABLE I) examined in this study were isolated from *Euwallacea* ambrosia beetles, avocado and other plants first by surface disinfecting them in 70% ethanol for 10 s followed by 0.01% sodium hypochlorite for 2 min before plating them on potato dextrose agar (PDA, Difco, Detroit, Michigan) in 100 × 20 mm plastic Petri dishes. Key isolates are available upon request from the Agricultural Research Culture Collection (NRRL) National Center for Agricultural Utilization Research, Peoria, Illinois (http://nrrl.ncaur.usda.gov/cgi-bin/usda/), NIAS Genebank, Microorganisms Section (MAFF) National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan, and the CBS-KNAW Fungal Biodiversity Center, Utrecht, the Netherlands (http://www.cbs.knaw.nl/collection/).

Molecular systematics and biology.—Methods for culturing mycelium, DNA extraction, PCR amplification and DNA sequencing followed protocols of O'Donnell et al. (2010). A rapid DNA extraction procedure also was employed for ap-PCR amplification. For this purpose, freshly harvested mycelium (100–200 mg) was placed in 2 mL tubes. Quantities of 700 µL breaking buffer (2% Triton-X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH = 8.0, EDTA 1 mM), 500 µL phenol/chlorophorm/isoamylalcohol (25 : 24 : 1) solution and two sterile metal beads (3 mm diam, SPEX Sample Prep, Metuchen, New Jersey) were added to each tube. Tubes were sealed and placed in a grinder (SPEX Sample Prep, New Jersey) for 90 s. The contents were centrifuged at 13 000 rpm for 20 min, and the supernatant was transferred to new tubes. Fifty microliters 3M Na acetate pH = 5.2 and 1 mL cold 100% ethanol was added to each tube. DNA was precipitated by centrifugation at 13 000 rpm for 10 min and washed twice with 70% ethanol. Pellets were dried in a laminar-flow chamber and resuspended in 50–100 µL TE buffer. A four-locus dataset was assembled that consisted of portions of translation elongation factor 1-α (*EF-1α*), DNA-directed RNA polymerase II largest (*RPB1*) and second largest subunit (*RPB2*) and nuclear ribosomal internal transcribed spacer region + domains D1 and D2 at the 5′ end of the nuclear large subunit (ITS + LSU rDNA) to assess evolutionary relationships among 18 isolates nested within Clade 3 of the FSSC. These isolates included representatives of six *Fusarium* species in addition to two isolates of *F. ambrosium* from galleries of *Euwallacea fornicatus* in *Camellia sinensis* (Chinese tea) in southern India, five of *F. euwallaceae* from avocado in Israel and one from avocado and *Quercus robur* (English oak) in Los Angeles County in southern California (TABLE I). DNA sequences were aligned with MUSCLE (Edgar 2004), adjusted manually, and model tests of the concatenated dataset (Posada 2008) selected GTR+Γ+I as the optimal model of molecular evolution. Clade support in the individual and combined datasets was assessed by maximum likelihood bootstrapping (ML-BS) in GARLI 1.0 (Zwickl 2006) run on the CIPRES Science Gateway site (Miller et al. 2010, http://www.phylo.org/portal2/login) and maximum parsimony bootstrapping (MP-BS) in PAUP* 4.0b10 (Swofford 2003). Arbitrarily primed PCR (ap-PCR) was performed on DNA of all the 145 tested isolates with three repeat-motif primers (i.e. $[CAG]_5$, $[GACAC]_3$, $[GACA]_4$) as reported in Freeman et al. (1993). DNA sequence data included in this study were deposited in GenBank as JQ038007–JQ038034.

Phenotypic characterization.—Strains were grown on PDA and synthetic low-nutrient agar (SNA; Nirenberg 1990, Nirenberg and O'Donnell 1998) in darkness, under continuous black light (National FL8BL-B 8W/08, Panasonic, Osaka, Japan), or under an ambient daylight photoperiod. Cultures on PDA in 9 cm Petri dishes at 20 C in darkness were used to characterize colony color, odor and morphology. Kornerup and Wanscher (1978) was used as the color standard. Cultures on SNA were employed for examination of microscopic characters as described by Aoki et al. (2005).

RESULTS

Molecular phylogenetics.—With the exception of the *EF-1a* partition that indicated *Fusarium ambrosium* and *F.* cf. *ensiforme* were sisters (FIG. 2A), MP-BS and ML-BS analyses of the *RPB1* (FIG. 2B), *RPB2* (FIG. 2C) and ITS+LSU rDNA (FIG. 2D) partitions and the combined dataset (FIG. 2E) strongly supported a sister group relationship between *F. ambrosium* and *F. euwallaceae* and their status as independent evolutionary lineages. Blue to brownish pigmented macroconidia was the only phenotypic character that was useful in distinguishing the latter two species. Pigmented macroconidia were common in all but one isolate of *F. euwallaceae*, whereas they were observed only in one isolate of *F. ambrosium* (NRRL 46583), where they were exceedingly rare. In addition to both ambrosia fusaria receiving strong phylogenetic support as independent evolutionary lineages, the different symbionts that comprise these obligate mutualisms support the recognition of *F. euwallaceae* (FIG. 3) as a new species of *Fusarium*. DNA typing 145 isolates of *F. euwallaceae* collected in the present study from several areas in Israel and California using the $(CAG)_5$ ap-PCR primer was concordant with the molecular phylogenetics, which suggested they likely comprised a highly clonal lineage (FIG. 3). DNA amplification with two additional ap-PCR primers, $(GACA)_4$ and $(GACAC)_3$, further supported their putative clonality (data not shown). Ap-PCR was able to discern *F. euwallaceae* from representative isolates of *F. ambrosium* from tea in India and a *Fusarium* sp. from *Ailanthus altissima* (Mill.) Swingle (tree-of-heaven) in Pennsylvania (FIG. 3), all of which are vectored by other *Euwallacea* spp. (Kasson et al. 2013).

TABLE I. Representative isolates of *Fusarium* used in this study

Isolate designations					
NRRL #[a]	Equivalent #[b]	Additional isolates[c]	Identified as[d]	Location	Host and cultivar (cv.)
54722*	Freeman 1	—	*F. euwallaceae*	Glil Yam, Israel	Live *Euwallacea* sp. IS/CA cv. Hass
54723	Freeman 2-1	—	*F. euwallaceae*	Glil Yam, Israel	Dead *Euwallacea* sp. IS/CA cv. Hass
54724	Freeman 2-11	4	*F. euwallaceae*	Mikve Yisrael, Israel	Dead *Euwallacea* sp. IS/CA cv. Hass
54725	Freeman 3-1	—	*F. euwallaceae*	Na'an, Israel	*Persea americana* cv. Hass
54726	Freeman 3-2	—	*F. euwallaceae*	Na'an, Israel	*Persea americana* cv. Ettinger
n/a[e]	Freeman 4-1	—	*F. euwallaceae*	Shiler Group, Israel	*Persea americana* cv. Hass
54727	Freeman 5-.4	1	*F. euwallaceae*	Volcani, Israel	*Persea Americana* cv. Hass
54728	Freeman 5-32	3	*F. euwallaceae*	Palmachim, Israel	*Euwallacea* sp. IS/CA gallery cv. Hass
n/a	Freeman 1-4	—	*F. euwallaceae*	Glil Yam, Israel	*Persea americana* cv. Hass
n/a	Freeman 5-1	8	*F. euwallaceae*	Palmachim, Israel	Live *Euwallacea* sp. IS/CA cv. Hass
n/a	Freeman 5-13	—	*F. euwallaceae*	Palmachim, Israel	Live male *Euwallacea* sp. IS/CA cv. Hass
n/a	Freeman 5-18	7	*F. euwallaceae*	Palmachim, Israel	Dead *Euwallacea* sp. IS/CA cv. Hass
n/a	Freeman 5-28	3	*F. euwallaceae*	Palmachim, Israel	Head of *Euwallacea* sp. IS/CA cv. Hass
n/a	Freeman 5-36	1	*F. euwallaceae*	Palmachim, Israel	*Euwallacea* sp. IS/CA larva cv. Hass
n/a	Freeman 6-1	—	*F. euwallaceae*	Volcani, Israel	Live *Euwallacea* sp. IS/CA from *P. americana* cv. Hass
n/a	Freeman 6-2	—	*F. euwallaceae*	Volcani, Israel	*Acer negundo*
n/a	Freeman 7-2	15	*F. euwallaceae*	Rishpon, Israel	Live *Euwallacea* sp. IS/CA from glue traps
n/a	Freeman 8-1	3	*F. euwallaceae*	Nachshon Israel	*Diospyros kaki*
n/a	Freeman 9-1	2	*F. euwallaceae*	Rishpon, Israel	*Persea americana*
n/a	Freeman 10-1	3	*F. euwallaceae*	Ga'ash, Israel	*Persea americana* cv. Hass
n/a	Freeman 11-1	—	*F. euwallaceae*	Shfa'im, Israel	*Persea americana* cv. Hass
n/a	Freeman 12-1	3	*F. euwallaceae*	Yakum, Israel	*Persea americana* cv. Hass
n/a	Freeman 13-1	5	*F. euwallaceae*	Tel-Aviv, Israel	*Acer negundo*
n/a	Freeman 13-4	1	*F. euwallaceae*	Tel-Aviv, Israel	Live *Euwallacea* sp. IS/CA from *A. negundo*
n/a	Freeman 14-1	2	*F. euwallaceae*	Beit-Dagan, Israel	Artificially infected *Acer negundo*
n/a	Freeman 16-1	—	*F. euwallaceae*	Ein-Vered, Israel	Live *Euwallacea* sp. IS/CA from *Ricinus communis*
n/a	Freeman 16-2	4	*F. euwallaceae*	Ein-Vered, Israel	*Ricinus communis*
n/a	Freeman 17-1	2	*F. euwallaceae*	Kibuz Eyal, Israel	*Persea americana* cv. Hass
n/a	Freeman 18-1	2	*F. euwallaceae*	Rishpon, Israel	*Ricinus communis*
n/a	Freeman 19-1	2	*F. euwallaceae*	Livnat, Israel	*Persea americana*
n/a	Freeman 24-1	5	*F. euwallaceae*	Israel	*Platanus orientalis*
n/a	Freeman 25-1	—	*F. euwallaceae*	Israel	*Ficus carica*
n/a	Freeman 26-1	2	*F. euwallaceae*	Beit Gamliel, Israel	*Quercus pedunculifolia*
n/a	Freeman 26-3	1	*F. euwallaceae*	Beit Gamliel, Israel	*Euwallacea* sp. IS/CA larva from *Quercus pedunculifolia*

TABLE I. Continued

Isolate designations					
NRRL #[a]	Equivalent #[b]	Additional isolates[c]	Identified as[d]	Location	Host and cultivar (cv.)
n/a	Freeman 26-6	—	*F. euwallaceae*	Beit Gamliel, Israel	Live *Euwallacea* sp. IS/CA from *Quercus pedunculifolia*
n/a	Freeman 27-1	4	*F. euwallaceae*	Nordia, Israel	*Acer negundo*
n/a	Freeman 28-1	2	*F. euwallaceae*	Even-Yehuda, Israel	*Quercus pedunculifolia*
n/a	Freeman 29-1	—	*F. euwallaceae*	Shiler Group, Israel	*Persea americana* cv. Ettinger
n/a	Freeman 29-2	—	*F. euwallaceae*	Shiler Group, Israel	*Persea americana* cv. Fuerte
n/a	Freeman 29-3	—	*F. euwallaceae*	Shiler Group, Israel	*Persea americana* cv. Hass
n/a	Freeman 34-1	—	*F. euwallaceae*	Na'an, Israel	*Persea americana* cv. Pino/TX
n/a	Freeman 34-2	—	*F. euwallaceae*	Na'an, Israel	*Persea americana* cv. Naor
n/a	Freeman 37-3	1	*F. euwallaceae*	Horshat-Tal, Israel	*Platanus orientalis*
n/a	Freeman 38-1	2	*F. euwallaceae*	Kibbutz Shamir, Israel	Live *Euwallacea* sp. IS/CA from *Acer negundo*
n/a	Freeman 38-4	2	*F. euwallaceae*	Kibbutz Shamir, Israel	*Acer negundo*
n/a	Freeman 39-1	—	*F. euwallaceae*	Regavim, Israel	*Quercus ithaburensis*
n/a	Freeman 41-1	—	*F. euwallaceae*	Regavim, Israel	*Persea americana* cv. Nabal
n/a	Freeman 42-1	—	*F. euwallaceae*	Hagoshrim, Israel	Live *Euwallacea* sp. IS/CA cv. Galil
n/a	Freeman 43-1	2	*F. euwallaceae*	Magal, Israel	*Persea americana*
n/a	1854	—	*F. euwallaceae*	California, USA	*Persea americana*
n/a	FD64	—	*F. euwallaceae*	California, USA	*Persea americana*
20438	IMI 296597	—	*F. ambrosium*	India	*Camellia sinensis*
22346	CBS 571.94	—	*F. ambrosium*	India	*Camellia sinensis*
62579	FRC S-2581	—	*Fusarium* sp.	Pennsylvania, USA	*Euwallacea validus ex Ailanthus altissima*
62580	FRC S-2581	—	*Fusarium* sp.	Pennsylvania, USA	*Euwallacea validus ex Ailanthus altissima*

[a] NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois). *, isolate used for holotype description.

[b] Collection of S. Freeman.

[c] Additional isolates that are identical to those from the same origin: Freeman 2-11, 2-12, 2-13, 2-14, 2-15; Freeman 57-4, 21-5; Freeman 5-32, 5-33, 5-34, 5-35; Freeman 5-1, 5-5, 5-6, 5-7, 5-8, 5-9, 5-10, 5-11, 5-12; Freeman 5-18, 5-20, 5-21, 5-22, -5-23, 5-24, 5-26, 5-27; Freeman 5-28, 5-29, 5-30, 5-31; Freeman 5-36, 5-37; Freeman 7-2, 7-3, 7-4, 7-5, 7-6, 7-7, 7-9, 7-11, 7-12, 7-13, 7-14, 7-15, 7-16, 7-17, 7-18, 7-19; Freeman 8-1, 8-2, 8-3, 8-4; Freeman 9-1, 9-2, 9-3; Freeman 10-1, 10-2, 10-3, 10-4; Freeman 12-1; 12-2, 12-3, 12-4; Freeman 13-1, 13-2, 13-3, 15-2, -15-3, 15-4; Freeman 13-4, 15-1; Freeman 14-1, 14-2, 14-3; Freeman 16-2, 16-3, 16-4, 16-5; Freeman 17-1, 17-2, 17-3; Freeman 18-1, 18-2, 18-3; Freeman 19-1, 19-2, 19-3; Freeman 24-1, 24-2, 24-3, 24-4, 24-5, 24-6; Freeman 26-1, 26-2; Freeman 26-3, 26-5; Freeman 27-1, 27-2, 27-3, 27-4, 27-5; Freeman 28-1, 28-2, 28-3; Freeman 37-3, 37-4; Freeman 38-1, 38-2, 38-3; Freeman 38-4, 38-5, 38-6; Freeman 43-1, 43-2, 43-3.

[d] Identified to species using multilocus sequencing or ap-PCR with multiple primers.

[e] Not available.

Fusarium euwallaceae S. Freeman, Z. Mendel, T. Aoki et O'Donnell, sp. nov. (FIGS. 4, 5)
MycoBank MB803293.

Colonies on PDA have radial mycelial growth rates of 3.1–3.7 mm/d at 20 C and 4.5–4.8 mm/d at 25 C in the dark. Colony color on PDA white (1A1) to yellowish white (4A2), pale yellow (4A3), light yellow (4A4) or orange-white (5A2), orange-gray (5B2), or often pale orange (5A3), sometimes reddish gray (11B2, 12B–C2), brownish gray (11C–D2), or purplish gray (13B–C2) in the dark, later with conidial pustules of yellowish white (4A2), pale yellow (4A3) to light yellow (4A4), grayish yellow (2–3B–C3) to grayish green (28–30B–D3–5, 28–30D–E5–7), and often dark green (25–30F5–8) when produced on sporodochia. Aerial mycelium sparse with pionnotal colony appearance, or developed abundantly, then loose to floccose, white (1A1) to yellowish white (4A2), or reddish gray (11B2, 12B–C2), brownish gray (11C–D2), or purplish gray (13B–C2). Colony margin entire

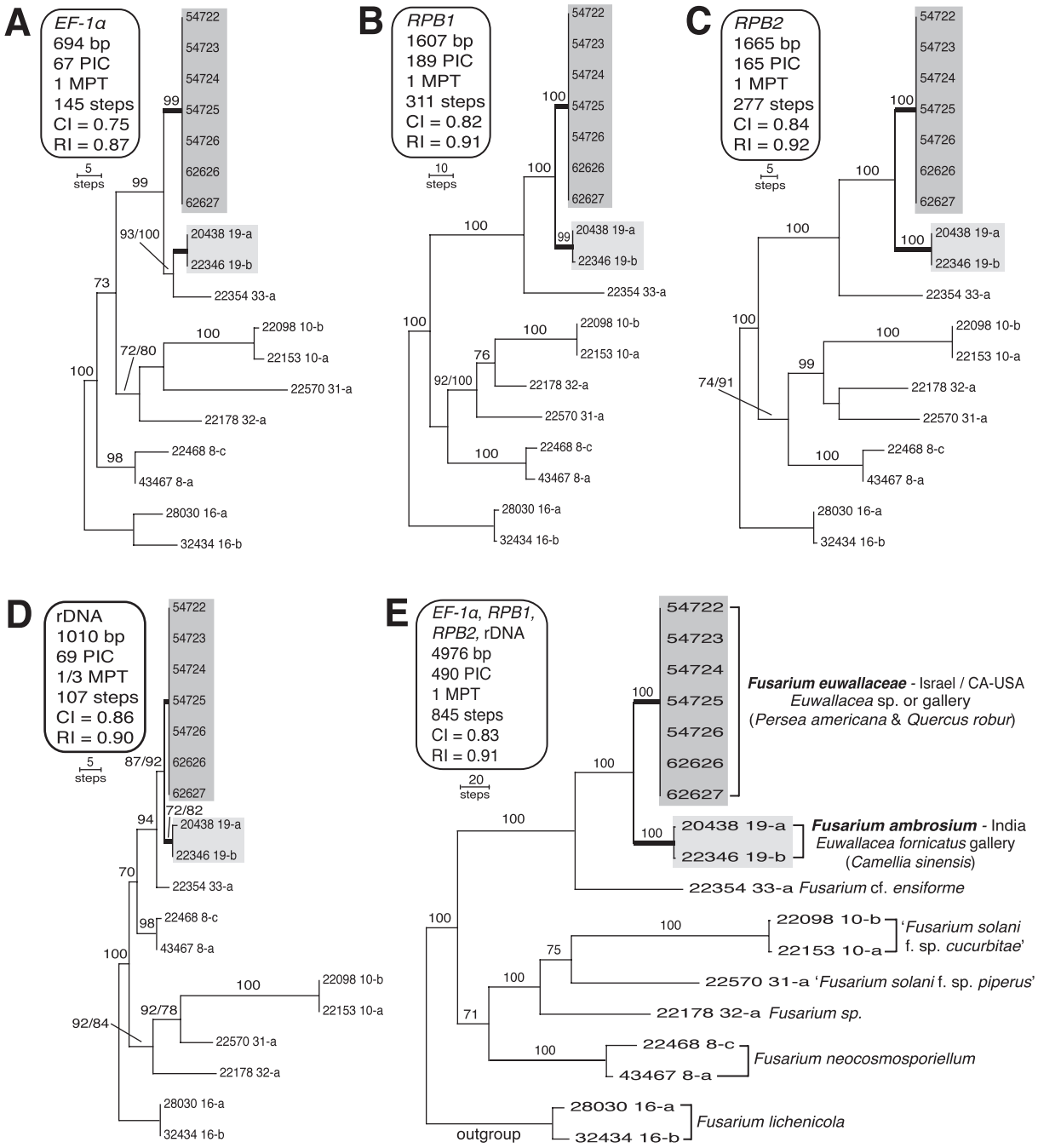


FIG. 2. One single most parsimonious phylogram illustrating phylogenetic relationships of *Fusarium euwallaceae* inferred from portion of four individual genes (A–D) and the combined four-gene dataset (E). A. Translation elongation factor (1-α). B. DNA-directed RNA polymerase II largest subunit (*RPB1*). C. DNA-directed RNA polymerase II second largest subunit (*RPB2*). D. Nuclear ribosomal internal transcribed spacer region and domains D1 + D2 of the nuclear large subunit (ITS+LSU) rDNA. E. Combined four-gene dataset. Numbers above nodes represent MP-BS/ML-BS based on 1000 pseudoreplicates of the data. The ML-BS value is not presented if it differed by <5% of the MP-BS value. Isolates of the two ambrosia fusaria, *F. euwallaceae* and *F. ambrosium*, are highlighted in gray and subtended by bold internodes. Each isolate is identified by a five-digit ARS Culture Collection (NRRL) number and, with the exception of *F. euwallaceae*, an informal FSSC species/haplotype (i.e. Arabic number/lowercase Roman letter) identifier (O'Donnell et al. 2008). CI = consistency index, MPT = most parsimonious tree, PIC = parsimony informative character, RI = retention index.

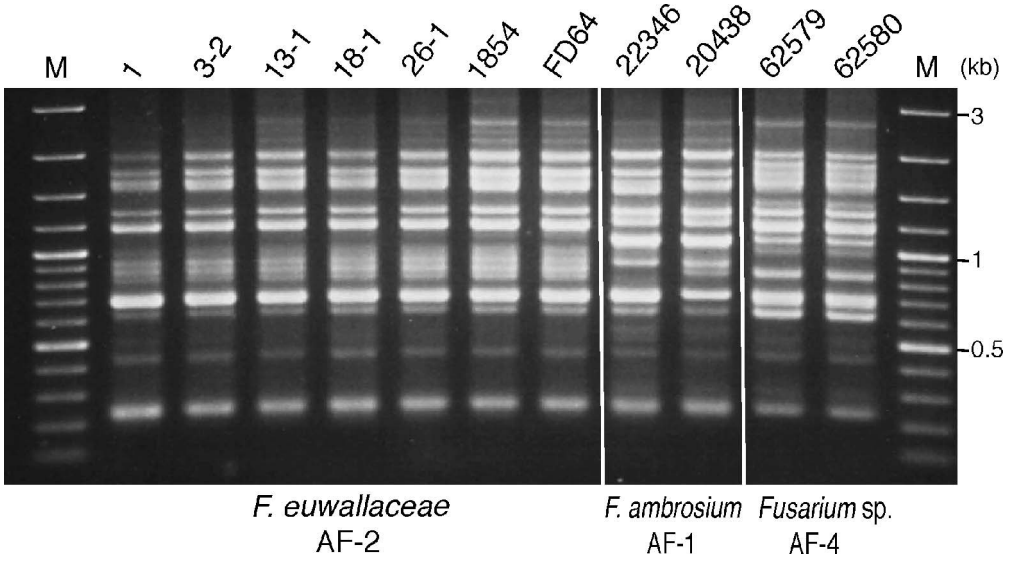


FIG. 3. Ap-PCR amplification of DNA from representative isolates of *Fusarium* spp. recovered from various *Euwallacea* ambrosia beetles using repeat-motif primer $(CAG)_5$. *Fusarium euwallaceae* (= AF-2) from the following regions and hosts: 1 and 3-2, Israel avocado; 13-1, Israel box elder; 18-1, Israel castor bean; 26-1, Israel oak; 1854, California avocado; FD 64, California avocado; *F. ambrosium* (= AF-1) 22346 and 20438 from tea in India and *Fusarium* sp. (= AF-4) 62579 and 62580 from tree-of-heaven in Pennsylvania. M, GeneRuler 100 bp plus DNA ladder molecular weight marker (Thermo Scientific, Waltham, Massachusetts) in kilobases (kb). Because several ambrosia *Fusarium* clade species are unnamed each species was given a unique AF designation (Kasson et al. 2013).

to undulate. Reverse pigmentation absent or pale yellow (4A3), grayish orange (5B3–4) to brownish orange (5C4–6) or sometimes with diffusing pigmentation of brown (6–7D–E4–6) to reddish brown (8–9D–F4–6). Colorless or orange to pinkish exudates sometimes observed. Odor sweet or moldy. Hyphae on SNA 1.5–9.5(–13.5) µm wide. Chlamydospores formed abundantly in hyphae and in conidia, mostly subglobose to round ellipsoidal, intercalary or terminal, single, or often in chains, ordinary hyaline to pale yellow, later becoming bluish to brownish when strongly pigmented, smooth to often rough-walled, 6–12 × 6–10 µm. Sclerotia absent. Sporulation on SNA generally rapid and abundant but slow in some strains, retarded on PDA; often light in the early stage on SNA and PDA in the dark or under daylight but later on PDA often forming abundant greenish sporodochia; sporodochia formed sparsely on SNA, but normally abundantly on PDA, sometimes less in mycelial colonies on PDA. Aerial conidiophores formed abundantly on SNA and PDA, erect, tall and narrow, mostly unbranched, rarely branched sparsely, up to 290 µm long, 3–5 µm wide at the base, thin-walled, forming monophialides integrated in the apices. Aerial phialides simple, subcylindrical to subulate, often with a conspicuous collarette at the tip. Aerial conidia mostly (1) ellipsoidal, fusiform-ellipsoidal to short clavate, occasionally reniform, 0–1(–2)-septate; 0-septate on SNA: 4.0–22.5 × 1.5–8.5 µm total range, 8.8–10.1 × 4.0–4.7 µm on average (ex type: 6.0–16.0 × 3.0–7.0 µm total range, 8.9 ± 1.9 × 4.3 ± 0.8 µm on average ± SD); one-septate on SNA: 5.5–28 × 2.5–9.5 µm total range, 9.6–17.0 × 4.5–6.7 µm on average (ex type: 8–24 × 4.5–8.5 µm total range, 15.2 ± 3.3 × 6.1 ± 1.1 µm on average ± SD) but sometimes formed together with (2) falcate to long clavate, sometimes curved cylindrical, (1–)2–3(–4)-septate conidia, morphologically almost indistinguishable from falcate sporodochial conidia. Sporodochial conidiophores short and thick, branched irregularly or unbranched, forming apical monophialides. Sporodochial phialides simple, subulate, lanceolate or subcylindrical, often with a conspicuous collarette at the tip. Sporodochial conidia mostly falcate to long clavate, sometimes curved cylindrical, swollen in upper parts, tapering toward the base, often with a round and papillate apical cell, and a distinct foot-like basal cell, conidia often with a dolphin-like appearance (Brayford 1987), (0–)3–4(–7)-septate, formed on PDA and SNA; three-septate on SNA in the dark: 21–45 × 6–11 µm total range, 28.3–36.5 × 8.3–9.4 µm on average (ex type: 22.5–39 × 6.5–11 µm total range, 30.6 ± 4.2 × 8.3 ± 0.8 µm on average ± SD), on SNA under black light: 21–56 × 5.5–11 µm total range, 30.7–38.5 × 8.2–9.4 µm on average (ex type: 23.5–44 × 6–10.5 µm total range,

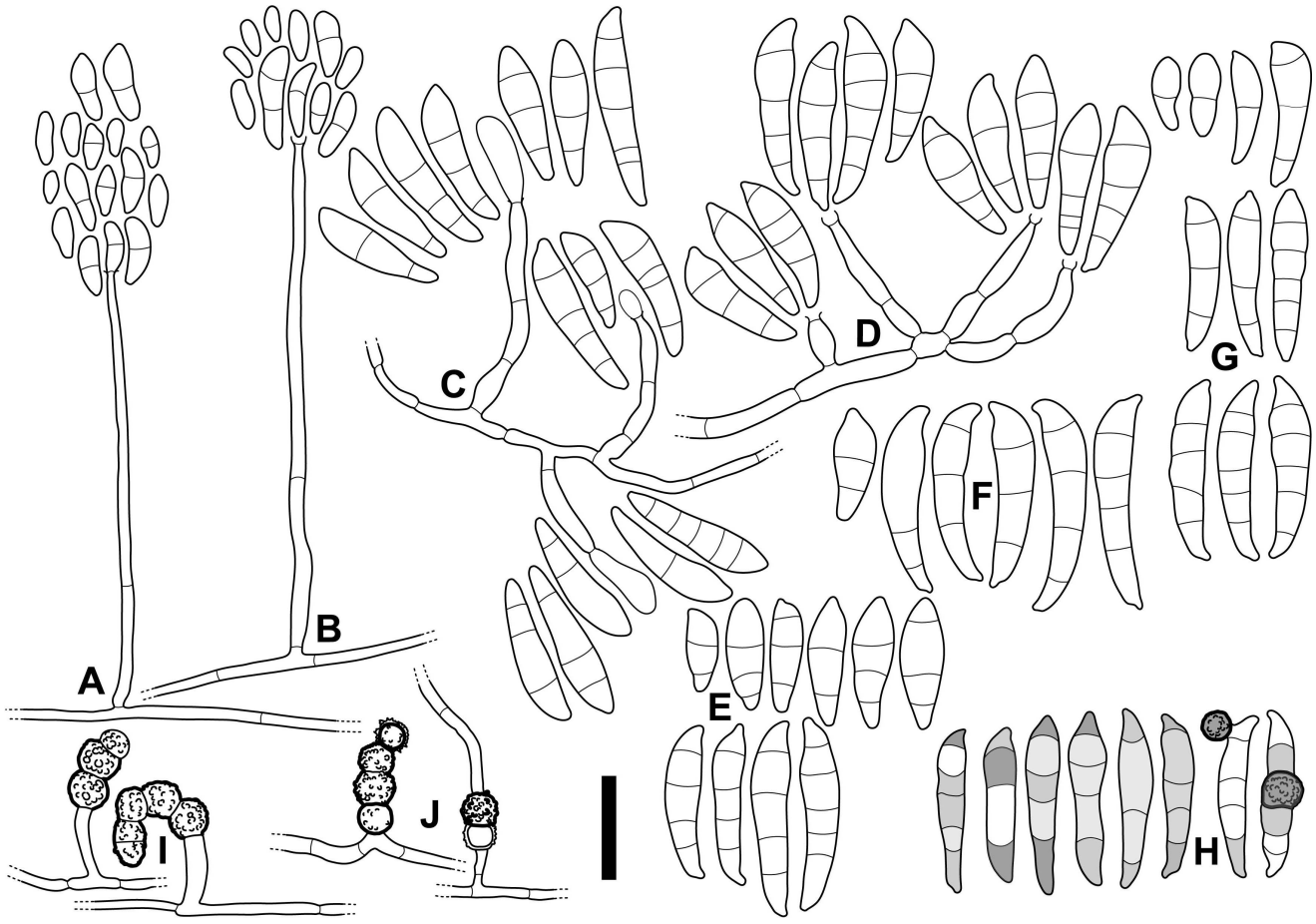
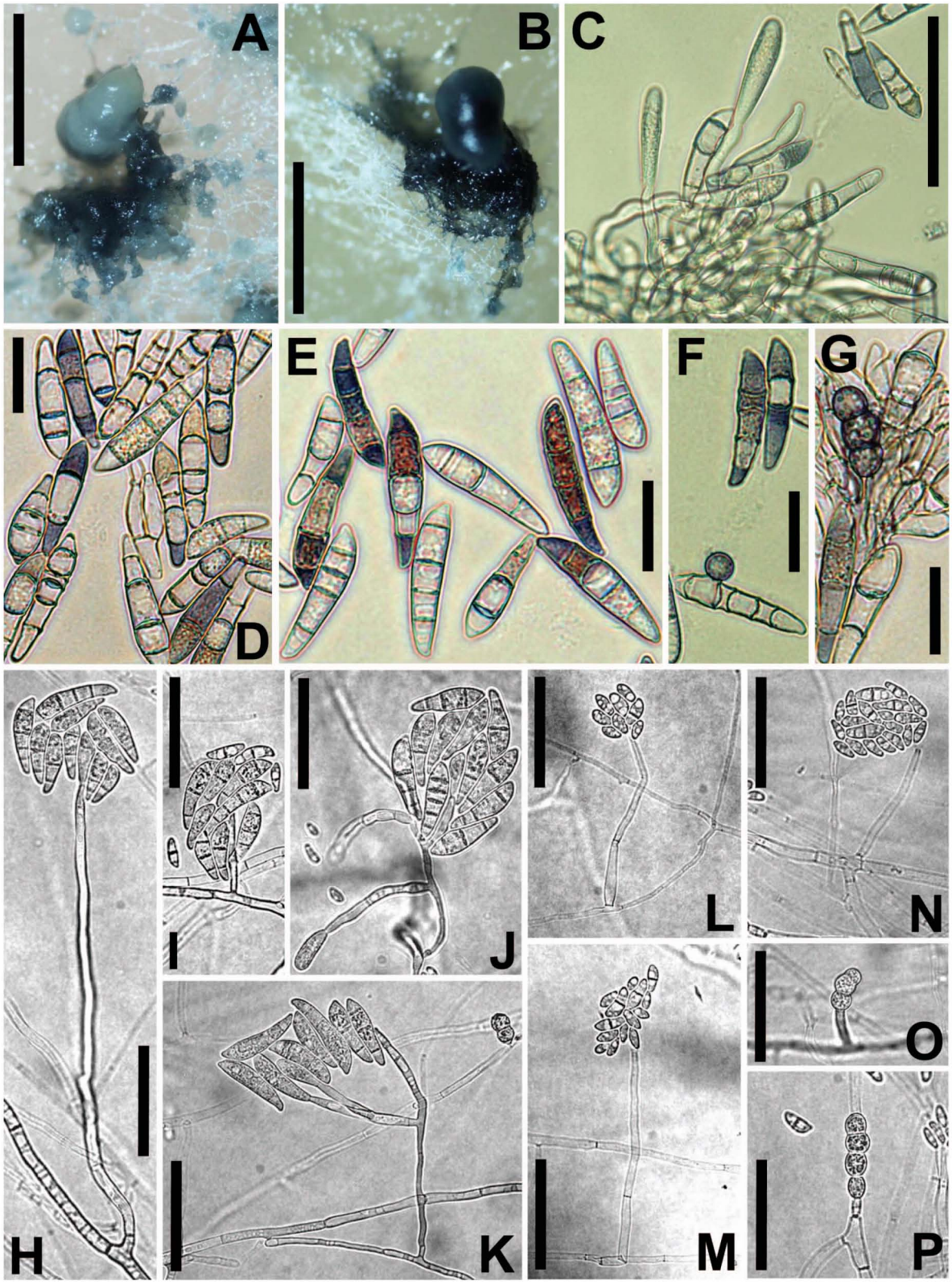


FIG. 4. Morphology of *Fusarium euwallaceae* cultured on synthetic low-nutrient agar (SNA) (A–F, I, J) and potato dextrose agar (PDA) (G, H). A, B. Long, stalked aerial conidiophores forming 0–1-septate, ellipsoidal to short clavate, occasionally reniform conidia with rounded ends. C, D. Shorter, simple or branched sporodochial conidiophores forming 1–5-septate, falcate to long clavate, sometimes curved cylindrical conidia, swollen in the upper half, tapering toward the base, often with a round and papillate apical cell and a distinct foot-like basal cell. E–G. 1–5-septate sporodochial conidia. H. Sporodochial conidia with bluish to brownish pigmented cells formed on sporodochia in greenish masses on PDA after one month. Rough-walled chlamydospores formed on and in conidia. I, J. Rough- and thick-walled chlamydospores formed in pairs or catenate. A, C, E–I from NRRL 54722 (ex holotype), B, D, J from NRRL 54726; A–C, E, G, I, J in dark, D, F under black light after 2 wk, H under daylight after 1 mo. Bar: 25 μm.

30.7 ± 4.9 × 8.2 ± 0.8 μm on average ± SD), on PDA in the dark: 17.5–47 × 5.5–10 μm total range, 27.0–35.0 × 7.3–8.2 μm on average (ex type: 27–47 × 6–9.5 μm total range, 34.1 ± 4.8 × 7.9 ± 0.6 μm on average ± SD); four-septate on SNA in the dark: 23.5–57.5 × 6–11 μm total range, 35.2–40.1 × 8.6–9.4 μm on average (ex type: 26.5–46.5 × 6–10 μm total range, 36.4 ± 4.2 × 8.6 ± 0.6 μm on average ± SD), on SNA under black light: 26–57.5 × 6–12 μm total range, 38.9–42.7 × 8.5–9.8 μm on average (ex type: 29–52 ×

→

FIG. 5. Morphology of *Fusarium euwallaceae* cultured on potato dextrose agar (PDA) under daylight after 1 mo (A–G) and synthetic low-nutrient agar (SNA) in dark after 2 wk (H–P). A, B. Greenish conidial masses formed on sporodochia in culture on PDA. C. Sporodochial phialides forming mutiseptate, falcate to long clavate conidia. D, E. Multiseptate, falcate to long clavate conidia, with swollen upper half, often with a round and papillate apical cell and tapering base. Bluish to brownish conidial cells formed in greenish conidial masses on PDA. F, G. Pigmented, rough-walled chlamydospores formed on conidium (F) and in hypha (G). H. Septate conidia formed on long-stalked aerial conidiophores. I–K. Septate conidia formed



on simple to branched, short sporodochial conidiophores. L–N. 0–1-septate, ellipsoidal to short clavate conidia formed on simple aerial conidiophores. O, P. Rough-walled chlamydospores in chains formed in hyphae. A, D, G, H-P from NRRL 54722 (ex holotype), B, C, F from NRRL 54728, E from NRRL 54726; bars: A, B = 1 mm, C = 50 µm, D–G = 20 µm, H–P = 50 µm.

7–11 µm total range, 39.7 ± 5.9 × 9.1 ± 0.9 µm on average ± SD), on PDA in the dark: 21.5–51 × 6–10.5 µm total range, 31.3–37.5 × 7.3–8.5 µm on average (ex type: 27.5–51 × 6.5–10.5 µm total range, 37.3 ± 4.7 × 8.1 ± 0.6 µm on average ± SD). Sporodochia conidia ordinarily hyaline but often with bluish to brownish pigmented cells when formed on sporodochia in greenish masses on PDA after a month; (2) oblong to naviculate or short-clavate, straight or curved, with a rounded apex and a truncate base, (0–)1(–2)-septate, formed sometimes together with multiseptate conidia.

Holotype: BPI 884203, a dried culture, was isolated from a living ambrosia beetle (*Euwallacea* sp. IS/CA) infecting avocado tree (*Persea americana* Mill.; Cultivar Hass) grown in an orchard at Kibbutz Glil Yam, central coastal region, Israel, 17 Feb 2010, by Stanley Freeman & Zvi Mendel (Freeman 1), and deposited in the herbarium of BPI (US National Fungus Collection).

Ex holotype culture: NRRL 54722 = MAFF 243811 = Freeman 1 = CBS 135854.

Etymology: euwallaceae, based on the generic name of the host ambrosia beetle, *Euwallacea*.

Isolates studied: NRRL 54722 = MAFF 243811 = Freeman 1 = CBS 135854; NRRL 54723 = MAFF 243812 = Freeman 2-1 = CBS 135855; NRRL 54724 = MAFF 243813 = Freeman 2-11 = CBS 135856; NRRL 54725 = MAFF 243814 = Freeman 3-1 = CBS 135857; NRRL 54726 = MAFF 243815 = Freeman 3-2 = CBS 135858; NRRL 54727 = MAFF 243816 = Freeman 57-4 = CBS 135859; NRRL 54728 = MAFF 243817 = Freeman 5-32 = CBS 135860; all isolated from live ambrosia beetles (*Euwallacea* sp.) infecting avocado tree, Kibbutz Glil Yam, Central coastal region, Israel, 17 Feb 2010, by Stanley Freeman and Zvi Mendel (TABLE I).

Notes: Fusarium euwallaceae is morphologically similar to *F. ambrosium* and several closely related undescribed species (Kasson et al. 2013). Similar to *F. euwallaceae*, *F. ambrosium* produces falcate to long clavate septate sporodchial conidia, swollen in their upper half, plus ovoid to ellipsoid, aseptate aerial conidia. Septate sporodochial conidia of *F. ambrosium*, for example, based on the study of NRRL 20438 and 22346 are three-septate on SNA in the dark: 22–4.0 × 6.0–11.0 µm total range, 29.5–30.6 × 8.5–8.6 µm on average; on SNA under black light: 20.5–52.0 × 4.5–11.5 µm total range, 34.1–35.8 × 8.3–9.1 µm on average; on PDA in the dark: 20.0–41.5 × 6.0–9.5 µm total range, 29.8–30.5 × 7.5–8.0 µm on average; four-septate on SNA in the dark: 25.0–44.0 × 7.0–10.5 µm total range, 35.6–35.8 × 8.2–8.8 µm on average; on SNA under black light: 27–53.5 × 7.0–12.0 µm total range, 41.0–41.3 × 9.0–10.1 µm on average; on PDA in the dark: 25.0–47.5 × 6–10 µm total range, 34.8–36.6 × 7.7–7.9 µm on average. Thus, conidial dimensions of these two fusaria are identical. Hence, *F. euwallaceae* can be characterized and differentiated from *F. ambrosium* by abundant bluish to brownish pigmented sporodochial conidia formed in greenish masses on PDA after a month (FIGS. 4H, 5A–G), in addition to hyaline conidia.

DISCUSSION

Because other fusaria associated with *Euwallacea* species produce highly modified clavate conidia (Kasson et al. 2013), which we think might indicate an adaption for the species-specific symbiosis, molecular phylogenetic data currently provide the most definitive method for distinguishing *F. euwallaceae* from related ambrosia congeners. The association with avocado, however, is not a reliable means for identifying *F. euwallaceae* because novel undescribed ambrosia fusaria have been recovered recently from this tree in southern Florida (R. Ploetz, J. Smith, J. Hulcr pers comm) and Queensland, Australia (A. Geering pers comm). In addition, this fungus causes deaths of a number of other host trees (Mendel et al. 2012, Eskalen et al. 2013). It seems unlikely that abundant production of bluish to brownish macroconidia (FIGS. 4H, 5A–G) by *F. euwallaceae* will serve as a reliable means for distinguishing this species from several closely related ambrosia fusaria. However, the fact that it takes approximately 1 mo to obtain morphology-based identification indicates that researchers interested in a rapid diagnosis will consider a molecular phylogenetic or ap-PCR-based approach.

The taxonomic history of *Fusarium ambrosium* is complicated because it originally was described as a species of *Monacrosporium* from *Euwallacea* (*Xyleborus*) *fornicatus* gallaries in *Camellia sinensis* (Chinese tea) stems in Sri Lanka (Gadd and Loos 1947) and subsequently redescribed as *F. bugnicourtii* (Brayford 1987) based on collections from galleries in Chinese tea in India and borer-damaged *Hevea brasiliensis* Müll. Arg. (rubber tree) and *Theoborma cacao* L. (cacao) in Malaysia. Brayford considered *F. bugnicourtii* to be conspecific with *F. tumidum* var. *coeruleum* Bugnicourt (Bugnicourt 1939) but distinct from *F. tumidum* Sherb. However, Bugnicourt (1939) based the type of *F. tumidum* var *coeruleum* on a collection from *H. brasiliensis*, which appears to be phylogenetically distinct from *F. bugnicourtii* = *F. ambrosium* (Kasson et al. 2013). Nirenberg (1990) synonymized *F. bugnicourtii* with *M. ambrosium* and recombined the latter as *F. ambrosium* based on nomenclatural priority. Authentic strains of *F. ambrosium* examined in the present study included NRRL 20438 = IMI 296597

isolated from *Euwallacea* (*Xyleborus*) *fornicatus* galleries in tea from India, which Brayford (1987) designated as the ex-holotype strain of *F. bugnicourtii*, and *F. ambrosium* NRRL 22346 = BBA 65390 = CBS 571.94, which was isolated from *Camellia sinensis* in India by V. Agnihothrudu and identified by H. Nirenberg (1990).

One intriguing question is whether *F. ambrosium* and *F. euwallaceae* differ in plant host range. Similar to *E. fornicatus*, *Euwallacea* sp. IS/CA attacks a wide variety of plants (Eskalen et al. 2012, Mendel et al. 2012). The host range of *E. fornicatus* sensu lato, which may comprise several cryptic species, was summarized by Danthanarayana (1968) from data accumulated from the tropics of Australasia and Indo-Malaya. Danthanarayana (1968) listed 99 host species belonging to 36 families; however, the beetles were able to breed in only 21 of the host plants. Future molecular phylogenetic studies are needed to assess whether Danthanarayana's list includes novel cryptic *E. fornicatus*-like species that vector different fusaria. For example *E. fornicatus* was reported from rubber trees (*Hevea brasiliensis*) in Malaysia (Malaya) (Kalshoven 1958); however, molecular phylogenetic analyses of a *Fusarium* isolated from this host suggest it is a unique species (Kasson et al. 2013). While avocado is a suitable host for *F. euwallaceae*, damage caused by *E. fornicatus* to this plant species in Sri Lanka has not been observed (K. Mohotti pers comm), further emphasizing the importance of a molecular approach to discern *Euwallacea* phylogeny, as well as the possible species complex in Sri Lanka.

Although inadvertent introduction of *Fusarium* into bark beetle galleries has been reported (Six 2003), *F. euwallaceae* consistently was isolated from live and recently dead beetles, as well as active galleries in live trees (Eskalen et al. 2012, 2013; Freeman et al. 2012a; Mendel et al. 2012). Furthermore, larvae reared on a diet of *F. euwallaceae* as their sole food source successfully developed into sexually competent females and males (Freeman et al. 2012b), whereas those reared on a diet of *F. ambrosium* did not. Similar to the findings of Freeman et al. (2012b), Norris and Baker (1967) reported that *Xyleborus ferrugineus* (Fabricius) could complete its life cycle only when fed a unique *Fusarium* species within the FSSC. Female *X. ferrugineus* produced galleries and fed extensively on the sterile medium of other fungi but required its symbiotic *Fusarium* to produce viable eggs, indicating that the symbiont synthesized compounds essential for reproduction of the beetle. Even though this finding suggests the symbionts are obligate mutualists, additional rearing experiments and cophylogeny studies are needed to critically assess whether transmission of ambrosia fusaria is strictly vertical (Mueller et al. 2005). In summary, this study was conducted to characterize and describe the novel species, *Fusarium euwallaceae*, a specific symbiont associated with the ambrosia beetle *Euwallacea* sp. IS/CA, affecting avocado and other host tree species in Israel and California.

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