Discordant phylogenies suggest repeated host shifts in the *Fusarium–Euwallacea* ambrosia beetle mutualism

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A R T I C L E   I N   P R E S S

Fungal Genetics and Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/fgb

ARTICLE INFO

Article history:
Received 28 August 2014
Accepted 27 October 2014
Available online xxxx

Keywords:
Cophylogeny
Fungiculture
Hybrid introgression
Molecular phylogenetics
Phylogenetic species
Symbiosis

A B S T R A C T

The mutualism between xyleborine beetles in the genus *Euwallacea* (Coleoptera: Curculionidae: Scolytinae) and members of the Ambrosia *Fusarium* Clade (AFC) represents one of 11 known evolutionary origins of fungicymbiosis by ambrosia beetles. Female *Euwallacea* beetles transport fusarial symbionts in paired mandibular mycangia from their natal gallery to woody hosts where they are cultivated in galleries as a source of food. Native to Asia, several exotic *Euwallacea* species were introduced into the United States and Israel within the past two decades and they now threaten urban landscapes, forests and avocado production. To assess species limits and to date the evolutionary diversification of the mutualists, we reconstructed the evolutionary histories of key representatives of the *Fusarium* and *Euwallacea* clades using maximum parsimony and maximum likelihood methods. Twelve species-level lineages, termed AF 1–12, were identified within the monophyletic AFC and seven among the *Fusarium*-farming *Euwallacea*. Bayesian diversification-time estimates placed the origin of the *Euwallacea–Fusarium* mutualism near the Oligocene–Miocene boundary ~19–24 Mya. Most *Euwallacea* spp. appear to be associated with one species of *Fusarium*, but two species farmed two closely related fusaria. *Euwallacea* sp. #2 in Miami-Dade County, Florida cultivated *Fusarium* spp. AF-6 and AF-8 on avocado, and *Euwallacea* sp. #4 farmed *Fusarium ambrosium* AF-1 and *Fusarium* sp. AF-11 on Chinese tea in Sri Lanka. Cophylogenetic analyses indicated that the *Euwallacea* and *Fusarium* phylogenies were largely incongruent, apparently due to the beetles switching fusarial symbionts (i.e., host shifts) at least five times during the evolution of this mutualism. Three cospéciation events between *Euwallacea* and their AFC symbionts were detected, but randomization tests failed to reject the null hypothesis that the putative parallel cladogenesis is a stochastic pattern. Lastly, two collections of *Euwallacea* sp. #2 from Miami-Dade County, Florida shared an identical cytochrome oxidase subunit 1 (CO1) allele with *Euwallacea validus*, suggesting introgressive hybridization between these species and/or pseudogenous nature of this marker. Results of the present study highlight the importance of understanding the potential for and frequency of host-switching between *Euwallacea* and members of the AFC, and that these shifts may bring together more aggressive and virulent combinations of these invasive mutualists.

Published by Elsevier Inc.

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http://dx.doi.org/10.1016/j.fgb.2014.10.014
1087-1845/Published by Elsevier Inc.

1. Introduction

The Fusarium–Euwallacea (Coleoptera: Curculionidae: Scolytinae) mutualism represents one of 11 known evolutionary origins of fungiculture by scolytine ambrosia beetles (Kasson et al., 2013). Euwallacea is a genus of over 40 species within the largest tribe of scolytine beetles, the Xyleborini, which contains nearly 1200 described species (Hulcr et al., 2015). All Xyleborini farm ambrosia fungi – symbionts that concentrate nutrients from their woody hosts. Most Xyleborini are associated with fungi from Ophiostomatales and Microascales (Beaver, 1989). Euwallacea is the only genus known to cultivate ambrosial fusaria, sometimes in addition to microscolean and ophiostomatalean fungi. The fusaria associated with Euwallacea form a monophyletic group (Ambrosia Fusarium Clade, or AFC) within the Fusarium solani species complex, originally reported to encompass 11 species lineages, nine of them (AF 1–9) with known associations with ambrosia beetles (Kasson et al., 2013). Published diversification-time estimates of the Xyleborini dated their evolutionary radiation to the early Miocene 21 Mya (Jordal and Cognato, 2012), similar to the inferred divergence time of the AFC (Kasson et al., 2013), suggesting that Euwallacea’s association with Fusarium, originated early after the origin of the whole fungus-farming tribe. Because there are no inferred evolutionary reversals to phloem-feeding, all of the fungus-farming beetles are thought to be obligate mutualists (Farrell et al., 2001). Female Euwallacea carry AFC mutualists in specialized mandibular cavities located within their mandibles termed mycangia (Beaver, 1989).

During the past two decades, several Euwallacea have been introduced from their native areas in Asia into Israel, Central America and at least five different locations within the United States (Eskalen and Stouthamer, 2012; Haack, 2006; Kasson et al., 2013; Kirkendall and Odregaard, 2007; Ploetz et al., 2013), presumably on infested wood packaging or plant material (Wingfield et al., 2010). The best-known example is the tea shot hole borer (TSHB), Euwallacea fornicatus Eichhoff, and its symbiont Fusarium ambrosium (Gaud and Loos) Agniproth. and Nirenberg. In their native region in southern Asia, these symbionts mostly colonize dead or declining species from at least 48 plant families (Danthanarayana, 1968; Hulcr et al., 2007), but they are also known as destructive pests of several economically important woody plants, including Chinese tea (Camellia sinensis) (L) Kuntze, avocado (Persea americana Mill.), citrus (Citrus spp.) and cacao (Theobroma cacao L.), where they can cause extensive dieback and even death (Brayford, 1987).

Recently, a new disease of avocado in California and Israel, Fusarium dieback, was initially reported to be associated with E. fornicatus (Eskalen et al., 2012; Eskalen and Stouthamer, 2012; Mendel et al., 2012). However, because preliminary molecular systematic data suggested the beetle in California and Israel was genetically distinct from E. fornicatus in Sri Lanka, it was given the common name polyphagous shot hole borer (PSHB) to distinguish it from the TSHB (Eskalen et al., 2013). Results of an extensive survey conducted in the aforementioned study revealed that the PSHB had attacked over 200 woody hosts representing 58 plant families in the Huntington Botanical Garden and Los Angeles Arboretum in the San Gabriel Valley of Southern California (Eskalen et al., 2013). In contrast to the TSHB that cultivates F. ambrosium on tea in India and Sri Lanka, the Euwallacea sp. in Israel and California farms a closely related species recently described as Fusarium euwallaceae (Freeman et al., 2013), which causes wilt and dieback of avocado and diverse landscape trees in concert with mass attack by the beetle (Eskalen et al., 2013). In this disease, the fungus moves from the beetle galleries along the xylem elements and adjacent cells, producing a brown discoloration and necrosis that often results in loss of yield, dieback and ultimately death in both young and mature trees (Mendel et al., 2012). Fusarium dieback of avocado caused little concern when it was first observed in Israel in 2005, but this disease is now responsible for serious damage mainly on avocado, box elder (Acer negundo L.), castor bean (Ricinus communis L.), and English oak (Quercus robur L.) in that country (Mendel et al., 2012). These four hosts are also among the 32 preferred by the PSHB in California (http://eskalen-lab.ucr.edu/avocado.edu.html), based on its ability to produce viable offspring on them (Eskalen et al., 2013). Although the PSHB was originally discovered in the Los Angeles Basin in 2003, the disease was not observed on avocado in California until 2012 (Eskalen et al., 2012) where it damaged the above hosts, as well as California coast live oak (Quercus agrifolia Née), California sycamore (Platanus racemosa Nutt.), and many other tree species (Eskalen et al., 2013). Conspicuous white eruptions of mannohexulose and perseitol referred to as ‘sugar volcanoes’ develop on affected avocado trees (see Fig. 2 in Mendel et al., 2012). These sugars are produced on branches and trunks of avocado in response to beetle entry and subsequent necrosis caused by F. euwallaceae (Freeman et al., 2013; Mendel et al., 2012).

Besides two separate infestations of E. fornicatus-like beetles in Southern California (i.e., San Diego County and Los Angeles and surrounding counties), and a third infestation in Miami-Dade County, Florida, two other exotic species of Euwallacea originally from Asia are now established in the US: Euwallacea validus Eichhoff and E. interjectus Blandford. E. validus is a temperate species with broad tree host range (Kabagla et al., 2006); a population increase has been documented on the invasive Asian tree of heaven (Ailanthus altissima (Mill.) Swingle) symptomatic for Verticillium wilt in eastern North America (Kasson et al., 2014). E. interjectus is a subtropical species spreading throughout the Southeastern US (Atkinson, 2014). Mass attack of this species has been documented on box elder in Gainesville, Florida (Kasson et al., 2013).

One of the most challenging aspects of research on the invasive Euwallacea sp. and their Fusarium symbionts has been defining taxonomic units, particularly species. The beetles are haplo-diploid inbreeders, which means that the great majority of matings occur within a family between a single haploid male and his diploid sisters (Kirkendall, 1993). Consequently, in the inbred Xyleborini, morphological characters change in a gradual fashion across clades, instead of being relatively uniform within species and different between species, as in most animals. Thus, applying a phylogenetic species concept within Euwallacea seems warranted given that the utility of traditional species concepts based on morphology or mating barriers remains to be determined. This is important in groups such as the E. fornicatus complex, where clades that are uniform morphologically may be distinct phylogenetically and ecologically, including different levels of aggression towards host trees. In this work, several of those populations are included, and are denoted only as Euwallacea sp., rather than E. fornicatus, in the absence of robust species-level molecular phylogenetic and type studies to determine what species represents the PSHB.

Similarly, the AFC fusaria have also evaded taxonomic treatment of species until recently (Freeman et al., 2013; Kasson et al., 2013). They comprise multiple morphologically cryptic species that are only known to reproduce asexually. In our previous molecular phylogenetic study of the AFC, F. ambrosium was the only species that had been described formally (Gaud and Loos, 1947; Nirenberg, 1990). Based on morphological differences from F. ambrosium and phylogenetics, F. euwallaceae was described for AFC–2, the AFC lineage associated with the Israeli and Los Angeles Basin outbreaks (Eskalen et al., 2013; Freeman et al., 2013).

To clarify the identity and evolutionary history of the globally invasive representatives of the Euwallacea–Fusarium symbiosis, the present study was conducted to (i) infer genealogically exclusive species boundaries (Taylor et al., 2000) by analyzing a 4-gene
dataset for the AFC and a 6-gene dataset for the Euwallacea that
cultivate them, (ii) date the evolutionary origin and diversification of
the Fusarium–Euwallacea mutualism, and (iii) conduct cophylo-
genetic analyses to determine the relative roles of cospeciation and/or host-shift speciation in the evolution of this increasingly
important mutualism.

2. Materials and methods

2.1. Taxon sampling

In contrast to males that lack mycangia and are flightless, female Euwallacea possess paired mandibular mycangia within which they transport their AFC symbionts (Kasson et al., 2013). Thus, to elucidate the fidelity of the Euwallacea–Fusarium mutualism, 103 adult female Euwallacea ambrosia beetles were collected at diverse localities within the United States, Israel, Sri Lanka, Papua New Guinea and Australia for DNA typing (Supplementary Table S1). Some beetles were obtained using sticky or funnel traps, but the majority were recovered directly from host trees. Once collected, beetles were either placed immediately in 95–100% ethanol or stored at –20 °C in the laboratory before shipping to NCAUR, Peoria, Illinois for DNA typing, or they were transported live to the laboratory. Fusaria were isolated from live-collected beetles by dilution plating whole beetles or heads that had been surface sterilized in 70% ethanol for 15 s and macerated in sterile water using a Tenbroeck tissue grinder (PYREX® No. 7727-07, Corning, NY). Macerates of each collection were serially diluted one, 10, and 100 times, and aliquots of the dilutions were spread on half-strength potato dextrose agar (½ PDA) amended with 100 µg ml⁻¹ of streptomycin sulfate (½PDA + SS, Sigma–Aldrich, St. Louis, MO) so that the number of viable AFC propagules could be quantified (Kasson et al., 2013). Once a head was removed with a sterile scalpel under a dissecting microscope, each thorax + abdomen was stored in a separate 1.5 ml Eppendorf tube containing 95–100% ethanol for subsequent DNA typing. The 91 fusaria that were DNA typed in the present study (Supplementary Table S2) are stored in 15% glycerol at -80 °C in the laboratory of K. O’Donnell at NCAUR, Peoria, IL. Fusaria that were analyzed phylogenetically using multilocus DNA sequence data are also available upon request from the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research (NCAUR), Peoria, Illinois USA (http://nrrl.ncaur.usda.gov/TheCollection/Requests.html). Other fungal symbionts were recovered from Euwallacea, including Raffaeleto and Graphium from E. validus (Kasson et al., 2013), but they were not included in this study because their presence was inconsistent in the various life stages examined.

2.2. Gene sampling

Total genomic DNA was isolated using an acetyl trimethyl-
ammonium bromide (CTAB; Sigma–Aldrich, St. Louis, MO) protocol from a whole female Euwallacea beetle or the thorax + abdominal region and from approximately 100 mg of freeze-dried mycelium of each AFC isolate that had been pulverized with a sterile pipette tip (O’Donnell et al., 1998). The generation of phylogenetic markers for both Euwallacea and AFC proceeded in two steps. First, the 103 Euwallacea individuals collected were typed using DNA sequence data from two markers: a portion of the nuclear large subunit 28S rDNA D2/D3 region (1004 bp alignment) and mitochondrial cytochrome oxidase subunit 1 (CO1) gene (689 bp alignment). DNA sequence data from three additional Xyleborus ambrosia beetle species (Cognato et al., 2011) were downloaded from GenBank for inclusion in the phylogenetic analyses of Euwallacea (Fig. 1, Supplementary Table S5). Relationships of the samples were inferred using a combined maximum parsimony phylogenetic analysis (MP, Swofford, 2003) of the 28S rDNA and CO1 sequences. Based on this phylogeny, 21 Euwallacea individuals from the three targeted species groups (E. formicatus, E. validus and E. interjectus) were selected to represent the range of phylogenetic diversity, and they were additionally typed using portions of four more genes (Table 1): translation elongation factor (EF-1α, 373 bp alignment), arginine kinase (ArgK, 669 bp alignment), CAD protein (CAD, 469 bp alignment), and mitochondrial 16S rDNA (16S rDNA, 420 bp alignment). PCR amplification and DNA sequencing followed published protocols (Dole et al., 2010; Simon et al., 1994; Supplementary Table S3).

Because two of the 103 Euwallacea individuals we typed appeared to represent E. validus – Euwallacea sp. #2 interspecific hybrids (Fig. 2; RP-30 and RP-31), the following steps were followed to rule out the possibility of technical error and cross-contamination of the samples with E. validus DNA. DNA was extracted separately from the two beetles using dedicated disposable pestles and PCR amplification and DNA sequencing of the nuclear and mitochondrial genes were repeated on three separate occasions. It is worth noting that the two putative E. validus – Euwallacea sp. #2 hybrid beetles were received in separate tubes filled with 100% ethanol from R. Ploetz’s lab where E. validus has never been processed. PCR primer pairs specific to the nuclear 28S rDNA, mitochondrial 16S rDNA and CO1 of Euwallacea sp. #2 and E. validus were designed and tested on genomic DNA from several E. validus and Euwallacea sp. #2 individuals, and the two putatively E. validus – Euwallacea sp. #2 hybrids. We reasoned that if the two putative hybrid samples had been contaminated with E. validus DNA, as opposed to only having an introgressed CO1 from E. validus, then the 28S and 16S rDNA of E. validus would have also amplified from these samples. The PCR assay specific to CO1 of Euwallacea sp. #2 also allowed us to assess whether the putative interspecific hybrids possessed an orthologous allele. The remaining combinations of primers and species were conducted as a positive control for the PCR.

Fusaria were initially typed using the intron rich 5’ end of EF-1α (687 bp alignment), which resolves all known species within the AFC (Kasson et al., 2013). Subsequently, for putatively novel species-level lineages within the AFC, portions of the following three nuclear genes were sequenced: rDNA internal transcribed spacer region (ITS rDNA) and 28S rDNA D1/D2 domains (1004 bp alignment), DNA-directed RNA polymerase II large (RPB1) subunit (1588 bp alignment), and DNA-directed RNA polymerase II second largest (RPB2) subunit (1635 bp alignment) (Supplementary Table S4). Multilocus DNA sequence data for 18 of the 25 fusaria included in the present study (Table 2) were published previously (Kasson et al., 2013).

As a prerequisite for our cophylogenetic analyses of the Fusarium–Euwallacea mutualism, phylogenetic species of the beetles and the fungi were delimited employing the operational criteria of genealogical concordance (GCPsR sensu Taylor et al., 2000) and non-discordance (Dettman et al., 2003). Phylogenetic species were recognized if bootstrapping of one or more of the individual partitions and the combined dataset supported their genealogical exclusivity at ≥70%, and their monophyly was not contradicted by bootstrap analyses of any of the individual gene partitions. DNA sequences generated in the present study have been deposited in GenBank under accession numbers KM406624–KM406751 and the alignments were deposited in TreeBASE as Tr77344, Tr77755, Tr77343, and Tr77754.

2.3. Phylogenetic analysis and diversification-time estimates

The individual and combined Euwallacea and Fusarium datasets were analyzed using maximum parsimony (MP, Swofford, 2002)
Fig. 1. (Left) One of nine most-parsimonious trees (MPTs) inferred from the combined four-gene data set (EF-1α, ITS + 28S rDNA, RPB1 and RPB2) for 12 Ambrosia Fusarium Clade (identified by red internode) species [AF-1 through AF-12]. Thickened black internodes identify Clades A and B within the Ambrosia Fusarium Clade. Color-coding of auxiliary lines are used to link nine fusaria with the Euwallacea sp. #4 from Chinese tea in Sri Lanka, cultivate two closely related fusaria. The putative fungus-farming xyleborine associated with AF-5 from Malaysia, AF-9 from Miami-Dade County, Florida and AF-10 from Singapore is unknown. (Right) Maximum parsimony analysis of a six-gene data set (ArgK, CAD, CO1, 16S rDNA and 28S rDNA) resolved seven fungus-farming Fusarium spp. Two Euwallacea spp., Euwallacea sp. #2 infesting avocado in Miami-Dade County, Florida and Euwallacea sp. #4 from Chinese tea in Sri Lanka, cultivate two closely related fusaria. The Euwallacea fornicatus Clade, which comprises six species lineages (Euwallacea sp. #1-6), is identified by a thickened black internode. Maximum likelihood (ML) and MP bootstrap (BS) values > 70%, based on 1000 pseudoreplicates of the data, are indicated above nodes (ML-BS/MP-BS). Only the ML-BS value is shown if it differed by <5% of the MP-BS value. The Fusarium and Euwallacea phylogenies were rooted on sequences of F. necrophorum (formerly known as Neocosmospora vasinfecta) and E. destruens + Wallacellus similis, respectively, based on more inclusive analyses (Cognato et al., 2011; O’Donnell et al., 2013). Divergence times were estimated with BEAST 2.1.3 (Bouckaert et al., 2014) using a birth–death model as the tree prior and an uncorrelated lognormal relaxed molecular clock. Each divergence time, including the median age (in bold) in millions of years before present (Mya) and the Bayesian 95% highest probability density interval, was taken from the chronograms presented in Supplementary Figs. S1 and S2. Photos at the top of the tanglegram illustrate club-shaped conidia of Fusarium sp. AF-7 NRRL 62610 from Australia and Euwallacea sp. #1 from Israel. CI, consistency index; MPTs, most-parsimonious trees; PIC, parsimony informative characters; RI, retention index. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
ascomycetous fungi (Kasson et al., 2013) were used to obtain a priori divergence dates for the BEAST analyses. Separate uncorrelated lognormal relaxed molecular clock analyses were conducted using BEAST ver. 1.6 (Drummond and Rambaut, 2007) was employed to compensate for potential differences in clade-specific rates. TRACER ver. 1.6 (Rambaut and Drummond, 2007) was used to determine that the Markov Chain Monte Carlo (MCMC) had reached convergence and Tree Annotator ver. 1.8.0 was used to summarize the results as maximum clade credibility (MCC) trees (Drummond and Rambaut, 2007; Supplementary Figs. S1 and S2). Statistical uncertainty in the divergence-time estimates was assessed by Bayesian Markov Chain Monte Carlo (MCMC) had reached convergence and Tree Annotator ver. 1.8.0 was used to summarize the results as maximum clade credibility (MCC) trees (Drummond and Rambaut, 2007; Supplementary Figs. S1 and S2). Statistical uncertainty in the divergence-time estimates was assessed by Bayesian

2.4. Cophylogenetic analysis of the Euwallacea–Fusarium mutualism

Jane 4 (Conow et al., 2010) and CoRe-PA (Merkle et al., 2010), two parsimony-based software tools, were used to reconstruct cophylogenetic scenarios, using the following predefined event cost values: speciation = 0, duplication = 1, host switch = 1, lineage sorting = 1, and failure to diverge = 1. The Euwallacea and Fusarium phylogenies were inferred, respectively, from datasets that consisted of sequences of the nine Euwallacea and fusaria that were isolated, we were able to obtain a preliminary estimate of their taxonomic identity remains to be determined. Six species level lineages within the morphospecies Euwallacea fornicatus are identified by #1–6. Euwallacea fornicatus was designated Euwallacea sp. #6 herein.

Table 1

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</table>

a The dataset consisted of portions of the following six genes: argK, EF-1α, CAD, cox1, 16S rDNA and 28S rDNA. Euwallacea ‘fornicatus’ was put in single quotes to indicate that it’s taxonomic identity remains to be determined. Six species level lineages within the morphospecies Euwallacea fornicatus are identified by #1–6. Euwallacea fornicatus was designated Euwallacea sp. #6 herein.


5

(EF-1x, ITS + 28S rDNA, RPB1 and RPB2) for the AFC that totaled 4.9 kb and comprised 388 parsimony informative characters (PIC) recovered nine equally most-parsimonious trees 566 steps in length. MP and ML methods resolved the same topology. The AFC ingroup was rooted using Fusarium neocosmosporiellum O'Donnell and Geiser (formerly Neocosmospora vasinfecta E.F. Sm.) as the outgroup based on more inclusive analyses of the EF-1α, RPB1 and RPB2 sequences of one to four of the seven E. fornicatus genealogies (Cognato et al., 2011) could not be tested, the other seven Euwallacea lineages were supported as genealogically exclusive by GCP-SR-based analyses of the individual and combined dataset (Fig. 1, right). With the exception of the 373 bp EF-1α gene partition that possessed too little phylogenetic signal to support the monophyly of any of the Euwallacea spp., bootstrap analyses of the five other individual partitions supported the monophyly of one to four of the seven Euwallacea lineages at > 70% (Table 4, Supplementary Table S7). As expected, bootstrapping the combined dataset provided the strongest phylogenetic hypothesis, given that 11 nodes and the seven Euwallacea lineages represented by more than one collection received strong (97–100%) bootstrap support (Fig. 1, Table 4).

Maximum likelihood and maximum parsimony analyses of a 23-taxon, 6-gene Euwallacea dataset (EF-1x, CAD, Arcg, 28S rDNA D2/D3, CO1 and 16S rDNA) that totaled 3.6 kb and contained 499 PIC found 180 MPTs 951 steps in length (Fig. 1, right; Table 4). The Euwallacea phylogeny used sequences of E. destruens Blandford and Wallacellus similis (Ferrari) from Papua New Guinea as outgroups, based on more inclusive analyses of the Xyleborini (Cognato et al., 2011). The three morphologically defined Euwallacea species analyzed resolved as strongly supported monophyletic groups (Fig. 1). The clade corresponding to the E. fornicatus morphology, however, was divided into five strongly supported species lineages and one single clade from Papua New Guinea. The six lineages comprising the E. fornicatus Clade (EFC) are heretofore referred to as Euwallacea sp. #1–6. The individual genes varied considerably in their contribution of phylogenetic signal, with CO1 (187 PIC) and the 28S rDNA D2/D3 regions (154 PIC) together contributing approximately two-thirds of the PIC, whereas EF-1α with 17 PIC contributed the least. Although the monophyly of a single collection from Papua New Guinea designated as Euwallacea sp. #10 from Singapore could not be tested because it was represented by a single, genetically divergent isolate, GCP-SR-based analyses of the individual and combined dataset supported the genealogical exclusivity of the other 11 AFC lineages (Fig. 1).

The combined analysis is presented as the strongest hypothesis of evolutionary relationships within the AFC in that 16 nodes and the 11 species represented by more than one isolate were strongly supported by ML and/or MP bootstrapping (Fig. 1, left; Table 3; Supplementary Table S6). Three putative novel AFC species were discovered in the present study: AF-10 (NRRL 62941 = IMI 351954) from an unknown host tree in Singapore, AF-11 (NRRL 62943 and 62944) from Chinese tea (Camellia sinensis) in Sri Lanka, and AF-12 (NRRL 62945 and 62946) from California sycamore (Platanus racemosa) and red willow (Salix laevigata Bebb) in San Diego County, California (Table 2). The latter species was resolved as a sister to Fusarium spp. AF-2, AF-3 and AF-4 with modest support, but no clear relationship between AF-10 and AF-11 and the other AFC lineages was inferred. With the addition of these taxa, the bootstrap support for the AFC and the two clades that comprise it (Clades A and B; Kasson et al., 2013; Fig. 1) remained very strong. Support for the backbone of AFC Clade B, which includes the TSHB and PSHB associates, F. ambrosium and F. euwallaceae, was weak as evidenced by four nodes that were not supported by ML and MP bootstrapping (Fig. 1).

(155 PIC) contributed similar phylogenetic signal, and together 80% of the PIC in the combined dataset, RPB2 outperformed RPB1 and the other genes in providing > 70% parsimony bootstrap support for nine of the 11 AFC species with more than one isolate. By contrast, phylogenetic analysis of the ITS + 28S rDNA revealed that it contributed the least phylogenetic signal with only 24 PIC; bootstrap analyses of this region only supported the monophyly of three AFC species (Table 3; Supplementary Table S6). Moreover, bootstrapping the ITS rDNA only resolved three of the 12 AFC species (AF-6, AF-8 and AF-9). Although the monophyly of Fusarium spp. AF-10 from Singapore could not be tested because it was represented by a single, genetically divergent isolate, GCP-SR-based analyses of the individual and combined dataset supported the genealogical exclusivity of the other 11 AFC lineages (Fig. 1).

Table 2

Histories of Ambrosia Fusarium Clade isolates included in the phylogenetic analysis (Fig. 1, left).

<table>
<thead>
<tr>
<th>Sequence ID</th>
<th>NRRL and equivalent</th>
<th>Xyloborus associate</th>
<th>Host/Substrate</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF-1 Fusarium ambrosium</td>
<td>NRRL 20438 = IMI 296597</td>
<td>E. formicarius</td>
<td>Camellia sinensis (tea)</td>
<td>India</td>
</tr>
<tr>
<td>AF-1 Fusarium ambrosium</td>
<td>NRRL 62942 = KOD 799 = PL-KH10</td>
<td>E. formicarius sp.</td>
<td>Camellia sinensis (tea)</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>AF-2 Fusarium euwallacea</td>
<td>NRRL 54727 = CBS 135859</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Israel</td>
</tr>
<tr>
<td>AF-2 Fusarium euwallacea</td>
<td>NRRL 62062 = AE-1854</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Los Angeles, CA</td>
</tr>
<tr>
<td>AF-3 Fusarium sp.</td>
<td>NRRL 62060 = CB-1499</td>
<td>E. interjectus</td>
<td>Acer negundo (box elder)</td>
<td>Gainesville-FL</td>
</tr>
<tr>
<td>AF-3 Fusarium sp.</td>
<td>NRRL 62060 = CB-1191</td>
<td>E. interjectus</td>
<td>Acer negundo (box elder)</td>
<td>Gainesville-FL</td>
</tr>
<tr>
<td>AF-4 Fusarium sp.</td>
<td>NRRL 62578 = FRC S-2576</td>
<td>E. validus</td>
<td>Atlantus altissima (tree-of-heaven)</td>
<td>PA-USA</td>
</tr>
<tr>
<td>AF-4 Fusarium sp.</td>
<td>NRRL 62579 = FRC S-2581</td>
<td>E. validus</td>
<td>Atlantus altissima (tree-of-heaven)</td>
<td>PA-USA</td>
</tr>
<tr>
<td>AF-5 Fusarium sp.</td>
<td>NRRL 22231 = IMI 110107</td>
<td>unknown</td>
<td>Hevea brasiliensis (rubber tree)</td>
<td>Malaysia</td>
</tr>
<tr>
<td>AF-5 Fusarium sp.</td>
<td>NRRL 46518 = FRC S-2075</td>
<td>unknown</td>
<td>Hevea brasiliensis (rubber tree)</td>
<td>Malaysia</td>
</tr>
<tr>
<td>AF-6 Fusarium sp.</td>
<td>NRRL 62590 = RP-AF9</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Miami, FL</td>
</tr>
<tr>
<td>AF-6 Fusarium sp.</td>
<td>NRRL 62591 = RP-16-1B</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Miami, FL</td>
</tr>
<tr>
<td>AF-7 Fusarium sp.</td>
<td>NRRL 62610 = AC-1</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Queensland, Australia</td>
</tr>
<tr>
<td>AF-7 Fusarium sp.</td>
<td>NRRL 62611 = AG-2</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Queensland, Australia</td>
</tr>
<tr>
<td>AF-8 Fusarium sp.</td>
<td>NRRL 62584 = RP-Amb2</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Miami, FL</td>
</tr>
<tr>
<td>AF-8 Fusarium sp.</td>
<td>NRRL 62585 = RP-AF4</td>
<td>E. euwallaceae sp.</td>
<td>Persea americana (avocado)</td>
<td>Miami, FL</td>
</tr>
<tr>
<td>AF-9 Fusarium sp.</td>
<td>NRRL 22643 = ATCC 44215</td>
<td>Xyleborus ferrugineus</td>
<td>Xyleborus ferrugineus</td>
<td>Costa Rica</td>
</tr>
<tr>
<td>AF-9 Fusarium sp.</td>
<td>NRRL 66088 = KOD 838</td>
<td>unknown</td>
<td>Delonix regia (royal poinciana)</td>
<td>Miami, FL</td>
</tr>
<tr>
<td>AF-10 Fusarium sp.</td>
<td>NRRL 62941 = KOD 147 = IMI 351954</td>
<td>unknown</td>
<td>unknown</td>
<td>Singapore, Malaysia</td>
</tr>
<tr>
<td>AF-11 Fusarium sp.</td>
<td>NRRL 62943 = KOD 796 = PL-KH1</td>
<td>E. euwallaceae sp.</td>
<td>Camellia sinensis (tea)</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>AF-11 Fusarium sp.</td>
<td>NRRL 62944 = KOD 797 = PL-KH2</td>
<td>E. euwallaceae sp.</td>
<td>Camellia sinensis (tea)</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>AF-12 Fusarium sp.</td>
<td>NRRL 62945 = KOD 793 = AE-FD420-G47</td>
<td>E. euwallaceae sp.</td>
<td>Platana racemosa (California sycamore)</td>
<td>San Diego, CA</td>
</tr>
<tr>
<td>AF-12 Fusarium sp.</td>
<td>NRRL 62946 = KOD 795 = AE-FD422-G69</td>
<td>E. euwallaceae sp.</td>
<td>Platana racemosa (California sycamore)</td>
<td>San Diego, CA</td>
</tr>
<tr>
<td>Fusarium neocosmosoriellum</td>
<td>NRRL 22468 = CBS 562.70</td>
<td>na</td>
<td>Stored peanuts</td>
<td>Guiné</td>
</tr>
<tr>
<td>Fusarium neocosmosoriellum</td>
<td>NRRL 43467 = CBS 139182</td>
<td>na</td>
<td>Human eye</td>
<td>LA-USA</td>
</tr>
</tbody>
</table>

a Genealogical concordance phylogenetic species recognition (Taylor et al., 2000) revealed that partial EF-1α can resolve all of the fusaria included in the present study except for AF-4 and AF-12.


c KOD, maintained in laboratory of Kerry O'Donnell, NCAUR, Peoria, IL; NRRL, ARS Culture Collection, Peoria, IL; PL, Pradeepa Liyanage, U. Colombo, Sri Lanka; RP, Randy Ploetz, U. Florida-Homestead.

d UIC, parsimony uninformative character.

e PIC, parsimony informative character.

Table 3

Statistics for the individual and combined Fusarium partitions.

<table>
<thead>
<tr>
<th>Locus</th>
<th># bp</th>
<th># MPTs</th>
<th>MPT length</th>
<th>CI</th>
<th>RI</th>
<th>UIC</th>
<th>PIC</th>
<th># Nodes/# species receiving ≥70% MP/ML-BSf</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF-1α</td>
<td>678</td>
<td>2</td>
<td>73</td>
<td>0.82</td>
<td>0.91</td>
<td>7</td>
<td>50</td>
<td>8/6</td>
</tr>
<tr>
<td>ITS + LSU rDNA</td>
<td>1004</td>
<td>36</td>
<td>38</td>
<td>0.89</td>
<td>0.93</td>
<td>8</td>
<td>24</td>
<td>4/3</td>
</tr>
<tr>
<td>RP1B1</td>
<td>1588</td>
<td>4</td>
<td>200</td>
<td>0.90</td>
<td>0.94</td>
<td>10</td>
<td>159</td>
<td>11/7</td>
</tr>
<tr>
<td>RP1B2</td>
<td>1635</td>
<td>366</td>
<td>227</td>
<td>0.74</td>
<td>0.85</td>
<td>8</td>
<td>155</td>
<td>10/9</td>
</tr>
<tr>
<td>Combined</td>
<td>4914</td>
<td>9</td>
<td>566</td>
<td>0.78</td>
<td>0.87</td>
<td>33</td>
<td>388</td>
<td>16/11</td>
</tr>
</tbody>
</table>

a MPTs, most-parsimonious trees.

b CI, consistency index.

c RI, retention index.

d UIC, parsimony uninformative character.

e PIC, parsimony informative character.

f Ones that received ≥70% maximum parsimony (MP) and/or maximum likelihood (ML) bootstrap support.

Table 4

Statistics for individual and combined Euwallacea partitions.

<table>
<thead>
<tr>
<th>Locus</th>
<th># bp</th>
<th># MPTs</th>
<th>MPT length</th>
<th>CI</th>
<th>RI</th>
<th>UIC</th>
<th>PIC</th>
<th># Nodes/# species receiving ≥70% MP/ML-BSf</th>
</tr>
</thead>
<tbody>
<tr>
<td>ArgK</td>
<td>669</td>
<td>&gt;50,000</td>
<td>82</td>
<td>0.89</td>
<td>0.94</td>
<td>33</td>
<td>146</td>
<td>5/2</td>
</tr>
<tr>
<td>CAD</td>
<td>469</td>
<td>&gt;50,000</td>
<td>64</td>
<td>0.89</td>
<td>0.93</td>
<td>20</td>
<td>35</td>
<td>3/2</td>
</tr>
<tr>
<td>EF-1α</td>
<td>373</td>
<td>&gt;50,000</td>
<td>37</td>
<td>0.89</td>
<td>0.92</td>
<td>16</td>
<td>17</td>
<td>1/0</td>
</tr>
<tr>
<td>LSU rDNA</td>
<td>1004</td>
<td>234</td>
<td>282</td>
<td>0.93</td>
<td>0.97</td>
<td>50</td>
<td>154</td>
<td>8/2</td>
</tr>
<tr>
<td>CO1</td>
<td>689</td>
<td>15,702</td>
<td>382</td>
<td>0.65</td>
<td>0.85</td>
<td>13</td>
<td>187</td>
<td>7/7</td>
</tr>
<tr>
<td>HDP rDNA</td>
<td>420</td>
<td>&gt;50,000</td>
<td>93</td>
<td>0.85</td>
<td>0.93</td>
<td>20</td>
<td>60</td>
<td>4/4</td>
</tr>
<tr>
<td>Combined</td>
<td>3624</td>
<td>180</td>
<td>951</td>
<td>0.79</td>
<td>0.90</td>
<td>124</td>
<td>499</td>
<td>11/7</td>
</tr>
</tbody>
</table>

a MPTs, most-parsimonious trees.

b CI, consistency index.

c RI, retention index.

d UIC, parsimony uninformative character.

e PIC, parsimony informative character.

f Ones that received ≥70% maximum parsimony (MP) and/or maximum likelihood (ML) bootstrap support.
Except for the branching order of *Euwallacea* spp. #2, #3 and #4, which was unresolved, the other nodes in the *Euwallacea* phylogeny received strong ML and MP bootstrap support (Fig. 1). Although *Euwallacea* spp. #1–5 and a specimen from Papua New Guinea designated *Euwallacea* sp. #6 matched *E. fornicatus* phenotypically (Cognato et al., 2011; NPAG, 2013), the present results suggest that they represent six phylogenetically distinct species. With the exception of *Euwallacea* sp. #6 that was missing CO1, the other *E. fornicatus*-like taxa were separated from one another by deep divergences (6.2–14.6%) in the CO1 gene tree (data not shown). While four of the *Euwallacea* species were associated with a single AFC species, AF-6 and AF-8 were recovered from *Euwallacea* sp. #2 on avocado in Miami-Dade County, Florida, and AF-1 and AF-11 were associated with *Euwallacea* sp. #4 on tea in Sri Lanka (Fig. 1).

Interestingly, multilocus sequencing of two *Euwallacea* sp. #2 individuals (i.e., RP-30 and RP-31) from avocado in Miami-Dade County revealed genealogical discordance between trees inferred from CO1 and the other loci. PCR assays using conserved and *Euwallacea* sp. #2-specific CO1 primers developed in the present study revealed that these two individuals possessed two divergent CO1 alleles. Although the partial nuclear 28S rDNA and mitochondrial 16S rDNA placed these collections in *Euwallacea* sp. #2, as did the partial ArgK, CAD and EF-1α sequences, the allele that was preferentially amplified in these two beetles with the conserved CO1 PCR primers (Dole et al., 2010; Supplementary Table S3) appeared to have been acquired from *E. validus* or an *E. validus*-like parent (Fig. 2). Alignment of the sequences from *E. validus* and the putative *E. fornicatus*-*E. validus* hybrids showed they shared an identical CO1 allele. In silico translations of the putative xenologous CO1 allele in these two collections suggested that it might be functional because the coding region was not interrupted by stop codons or indels. Results of PCR assays specific to the nuclear 28S rDNA, mitochondrial 16S rDNA and CO1 of *Euwallacea* sp. #2 and *E. validus* indicated that the two putative *E. validus*—*Euwallacea* sp. #2 hybrids only possessed orthologous 28S and 16S rDNA sequences, supporting the interpretation that the *E. validus* CO1 allele in these samples was the result of introgressive hybridization rather than cross-contamination with *E. validus* DNA. Furthermore, PCR assay with *Euwallacea* sp. #2 CO1-specific PCR primers revealed that the two hybrid beetles also possessed an orthologous CO1 allele, in addition to the xenolog (Fig. 2, Supplementary Table S3).

### 3.2. Divergence-time estimates of the *Euwallacea*-Fusarium mutualism

Divergence times for the AFC were estimated with BEAST ver. 2.1.3 (Boukaert et al., 2014) using external calibration points as previously described (Kasson et al., 2013; O’Donnell et al., 2013). The Bayesian analysis suggested the AFC evolved in the late Oligocene ~24 Mya [95% HPD interval: 13.9–34.5; Supplementary Fig. S1] (Fig. 1, left), followed by a basal split between Clades A and B in the middle Miocene 9 Mya [95% HPD interval: 1.4–18.4 Mya]. Of the 10 later diverging lineages for which divergence times were obtained, seven were dated to the Pliocene 2.3–5.1 Mya and three to the Pleistocene 0.6–1.8 Mya (Fig. 1). The poorly resolved backbone of the AFC Clade B phylogeny, together with the short internodes supporting the 10 fusaria within it, suggest they underwent a relatively rapid diversification over the past 5 million years.

Bayesian-derived divergence time estimates were obtained for *Euwallacea* and related Xyleborini with BEAST ver. 2.1.3, using fossil calibrations previously reported for the Scolytinae (Jordal and Cognato, 2012). Minimum (crown) time estimates suggest the AFC-farming *Euwallacea* diverged in the early Miocene 19.3 Mya [95% HPD interval: 10.7–28.2 Mya; Supplementary Fig. S2] (Fig. 1, right). The early diverging *E. validus* and *E. interjectus* lineages were dated to the mid-Miocene and radiance of the most recent common ancestor of the remaining ingroup to the late Miocene 6.5 Mya [95% HPD interval: 2.8–11.2 Mya]. All subsequent cladogenetic events were dated to the Pliocene 2.3–3.2 Mya and Pleistocene 1.5 Mya [95% HPD interval: 0.5–3.1 Mya].

### 3.3. Cophylogenetic analyses of the *Euwallacea*-Fusarium mutualism

To explore the relative roles of cospeciation, host-switching, duplication, and lineage sorting on a macroevolutionary scale, cophylogenetic analyses of multilocus DNA sequence data from nine *Euwallacea* representing seven species and the nine AFC they cultivate were conducted with two maximum parsimony tree reconciliation tools, Jane 4 (Conow et al., 2010) and CoRe-PA (Merkle et al., 2010). Jane 4 found one solution that had a maximum parsimony (i.e., minimum cost) solution of cost 5, with three cospéciations and five host switches (Fig. 3). CoRe-PA analysis resulted in the same reconciliation as well as another that differed only in that one of the host shifts was initially to *F. ambrosium* AF-1 followed by one to AF-11. CoRe-PA also found a third reconstruction of cost 5, but it was determined to be temporally invalid.

Since estimating appropriate event costs for maximum parsimony reconciliation is notoriously difficult, we also used the costscape tool in the Xscape software toolkit (Libeskind-Hadas et al., 2014) to explore the effects of a wide range of event costs (cospeciation cost fixed to 0, duplication normalized to 1, and host switch and loss ranging from 0.2 to 5). The costscape tool reported the number of evolutionary events in all maximum parsimony solutions over this cost range as well as the number of distinct optimal reconciliations. Costscape represents a maximum parsimony solution with an event count vector of the form <0,4,1> indicating the number of cospéciations, duplications, host switches, and losses, respectively. One group of solutions had event count vector <3,0,5,0> and there were two distinct reconstructions in this group – both found by CoRePA and one found by Jane (Fig. 3). Another maximum parsimony solution had event count vector <4,0,4,1>. Three other groups of solutions had event count vectors of <4,2,1>, <3,4,1,20>, and <3,5,0,27>. However, the latter three solutions are only optimal at extreme values for the costs of host switch or loss event and thus are unlikely to be of interest (Fig. 4). To assess whether the *Euwallacea*-Fusarium trees were similar due to chance, a permutation test employing 1000 trials was conducted using the sigescape tool in Xscape. When host switch and loss costs ranging from 1 to 5 were analyzed, 100% of the event cost space found by sigescape was not significant at the 0.05 level (data not shown). Thus, the null hypothesis that the three cospéciations could have occurred by chance could not be rejected.

### 4. Discussion

#### 4.1. Phylogenetic diversity of the Fusarium–Euwallacea mutualism

In our previous study (Kasson et al., 2013), we documented the phylogenetic diversity of fusaria engaged in the mutualistic association with *Euwallacea*. In this work we extended the study and discovered a similar pattern of apparent cryptic speciation among the ambrosia beetles that farm them. In *Fusarium*, we have now detected 11 species according to the criteria of genealogical concordance and non-discordance (GCPSR, Taylor et al., 2000; Dettman et al., 2003). These 11 reciprocally monophyletic species lineages were supported by bootstrap analyses of one or more of the single-gene genealogies and the combined dataset. Within the sampled *Euwallacea*, we detected seven phylogenetically distinct species lineages using the same criteria, five of them within
the morphological concept of *E. fornicateus*. Although the taxonomic status of the single isolate of *F. sp. AF-5* recovered from a beetle-infested rubber tree (*Hevea brasiliensis* Müll. Arg.) in Malaysia and an ambrosia beetle identified as *E. fornicateus* from Papua New Guinea (*Cognato et al., 2011*) could not be evaluated employing CCPSR-based criteria, they very likely represent phylogenetically distinct species based on the level of genetic divergence from their sister clades. Two additional findings support our interpretation of species diversity within the AFC and the *Euwallacea* spp. that cultivate them: (i) unique AFC symbionts are cultivated by each of the seven *Euwallacea* lineages, suggesting symbiosis-related adaptations towards maintaining vertical transmission, and (ii) the divergence-time estimates indicate the youngest species pairs we sampled within *Fusarium* and *Euwallacea* diverged ~0.6 and 1.5 Mya, respectively. The three fusaria discovered herein produced distinctive club-shaped macroconidia similar to those produced by six other members of the AFC (see Fig. 3 in Kasson et al., 2013). We posit that these spores represent a symbiosis-related adaptation, analogous to nodules, gongylidia or swollen cells produced by fungi that are cultivated, respectively, by termites (*Aanen et al., 2002*), leaf cutting ants (*Schultz and Brady, 2008*), and ambrosia beetles associated with Ophiostomatales or Microascales (*Massoumi Alamouti et al., 2009*).

The number of ambrosial *Fusarium* species was extended from 9 to 12 in this study by the discovery of AF-11 – *Euwallacea* sp. #4 on tea in Sri Lanka, AF-12 – *Euwallacea* sp. #5 on California sycamore and red willow in San Diego County, California, and the singleton AF-10 from Singapore. The provenance of the previously identified AF-9 remains uncertain. This taxon (= NRRL 22643 received as ATCC 44215 *F. solani* (*Norris, 1980*)) is a phylogenetically distinct member of the AFC that the ATCC catalog indicates was isolated from *Xyleborus farrugineus* in Costa Rica. However, because the beetle used in the Norris publication was not identified, and *Euwallacea* and *Xyleborus* are not reciprocally monophyletic (*Cognato et al., 2011*), the identity of the xyleborine beetles reared in these studies (*Norris and Baker, 1967*) remains to be determined. Beetle vouchers were not deposited in the Wisconsin Insect Research Collection at the University of Wisconsin–Madison (Daniel Young, pers. comm.). An isolate of AF-9 (Table 2) was also recovered in the
present study from beetle-infested royal poikincana, Delonix regia (Boj. ex HOOK.) Raf., in Miami-Dade County, Florida, a known host of an ambrosia beetle reported as *E. fornicatus* (Thomas, 2012). However, further work is needed to ascertain whether AF-9 is cultivated by the *Euwallacea* ambrosia beetle reported to infest this host. Future studies are also needed to match AFC species AF-5 from a beetle-damaged rubber tree (*H. brasiliensis*) in Malaysia and AF-10 received as IMI 351954 *Fusarium bugnicourtii* Brayford (a later synonym of *F. ambrosium*; Nirenberg, 1990) from an unknown woody host in Singapore.

Six of the eight *Euwallacea* lineages analyzed here fit the morphological diagnosis of *E. fornicatus*, the TSHB from Sri Lanka and India: (1) *Euwallacea* sp. #1 from avocado and diverse woody hosts in California and Israel (Eskalen et al., 2012; Mendel et al., 2012), (2) *Euwallacea* sp. #2 from avocado in Miami-Dade County, Florida, (3) *Euwallacea* sp. #3 from avocado in Queensland, Australia, (4) *Euwallacea* sp. #4 from Chinese tea in Sri Lanka, (5) *Euwallacea* sp. #5 from red willow and California sycamore in San Diego County, California, and (6) ‘*E. fornicatus*’ from Papua New Guinea, in which the fungal symbiont was not identified (Cognato et al., 2011). The taxonomic identity of the actual *E. fornicatus* is currently unclear, as the type specimen has been lost from the museum where it was originally deposited. The morphological crypts observed here for *Euwallacea* is consistent with that reported for numerous other Xyloborini, and may be explained by lack of strong selection on secondary sexual characters (Farrell et al., 2001; Jordal et al., 2000) and by the relatively recent radiation of the six *E. fornicatus*-like taxa over the past 6.5 Ma (Fig. 1, right). Consistent with their obligatory sib-mating and haplodiploidy, multilocus phylogenetic analyses revealed little allelic variation within the seven highly inbred *Euwallacea* spp. we sampled.

Results of the present study highlight the need for more informative loci for species-level studies within *Euwallacea* and the AFC. While EF1-α, CAD and ArgK have phylogenetic utility at higher taxonomic levels within various Coleoptera (Cognato et al., 2011; Wild and Maddison, 2008), our results indicate that these three genes have very limited utility for species-level studies of *Euwallacea* that cultivate AFC. Similarly, of the four unlinked genes sequenced in the AFC, the fungal barcode locus ITS rDNA and D1/ D2 region of the 28S rDNA contained the least phylogenetic signal in that it only contributed 24 phylogenetically informative characters. The availability of a whole genome sequence of *F. euwallaceae* NRRL 62626 (Stajich and O’Donnell, unpubl.), and one of the closely related pea pathogen, *F. solani* (Mart.) Sacc. f. sp. *pisum* W.C. Snyder and H.N. Hansen (Coleman et al., 2009), should provide a wealth of phylogenetically informative loci for species-level studies within the AFC.

Preliminary evidence was obtained consistent with hybrid introgression and parental leakage of the CO1 gene of *E. validus* – *Euwallacea* sp. #2 hybrid beetles (RP-30 and RP-31) were received in separate tubes filled with 100% ethanol from R. Ploetz’s lab where *E. validus* has never been processed. The xenologous origin of the *E. validus* CO1 allele in two *Euwallacea* sp. #2 individuals from Miami-Dade County, Florida was confirmed by novel PCR assays specific to the nuclear 28S rDNA, mitochondrial 16S rDNA and CO1 from the two ambrosia beetle species. These assays revealed that the two putative hybrids only possessed orthologous 28S and 16S rDNA sequences, supporting the interpretation that the *E. validus* CO1 allele in these samples was the result of introgressive hybridization rather than cross-contamination with *E. validus* DNA. We were also able to establish, via a PCR assay with *Euwallacea* sp. #2 CO1-specific PCR primers, that the two hybrid individuals possess an orthologous allele. Even though the putative xenologous CO1 allele of the hybrids was not interrupted by stop codons and indels (i.e., cryptic sensu Berttheau et al., 2011), suggesting that it might be functional, additional studies are needed to determine whether it represents a pseudogene that integrated into the mitochondrial or nuclear genomes of *Euwallacea* sp. #2, or both. Assuming the *E. validus* CO1 allele is present within the mitochondria, then the paternally and maternally inherited mitochondrial genomes may have recombined because the 16S mitochondrial rDNA was inherited from *Euwallacea* sp. #2. Efforts are being made to obtain additional samples of the putative *E. validus* – *Euwallacea* sp. #2 hybrid so that it can be subjected to a whole genome analysis.

Paternal inheritance of mitochondrial DNA has been reported in diverse insects (Arunkumar et al., 2006; Meusel and Moritz, 1993; Sherengul et al., 2009; Wolff et al., 2013; Zakharov et al., 2009), other animals including humans (Bromham et al., 2003; Kvist et al., 2003; Zhao et al., 2004; Zouros et al., 1994) and plants (Neale et al., 1989). Given the well-documented cases of introgression of mtDNA (Ballard, 2000; Jordal and Kambestad, 2013; Shaw, 2002), and Wolbachia infections in diverse arthropods (Werren et al., 2008), including ones we detected in *E. validus* and *Euwallacea* sp. #2 (O’Donnell, unpubl.) and other xyloborine beetles (Kawasaki et al., 2010), it is worth determining whether the putative hybrid introgression of mitochondrial DNA we discovered was driven by Wolbachia-infected *Euwallacea* Hu (Jettis and Jiggins, 2004).

Of the 10 independent evolutionary origins of fungus-farming that have been documented in the weevil subfamily Scolytinae, the bark and ambrosia beetles (Jordal and Cognato, 2012), only one appears to involve the fungiculture of *Fusarium* – that observed in the xyloborine genus *Euwallacea*. All other known ambrosia beetles engage in mutualism with symbionts that belong to other fungal genera, mostly from Ophiostomatidae and Microscales (Massoni Almouati et al., 2009). The available data suggest that this mutualistic symbiosis arose monophyletically in the late Oligocene to early Miocene ~19–24 Mya (Kasson et al., 2013; present study). *Euwallacea* is a derived genus within Xyloborini, and possesses the ancestral mandibular mycangium (Cognato et al., 2011). Therefore its ancestors probably already engaged in the ambrosia symbiosis, but with different fungi. The observation of
Raffaelea (Ophiostomataceae) and Graphium (Microascales) spp. in association with E. validus (Kasson et al., 2013) may reflect a partial retention of this ancestral character.

Prior to the outbreak of Fusarium dieback in California (Eskalen et al., 2013) and Israel (Mendel et al., 2012), the best studied example of Fusarium fungivory was the tea shoot hole borer (TSHB), E. fioriticus (formerly known as Xyleborus fioriticus), which cultivates F. ambrosium in Chinese tea in Sri Lanka and India (Brayford and Gadd, 1947). Fusaria were noticeably absent, however, from two key prior phylogenetic analyses of ambrosia fungi (Massoumi Alamouti et al., 2009; Farrell et al., 2001) that included mostly ophiostomatoid and to a lesser extent microascalean fungi. Fusaria have been recovered from numerous phylogenetically diverse insects (O’Donnell et al., 2012), including wood-boring beetles, but the nature of these associations are largely unknown. The Asian longhorned beetle, Anoplophora glabripennis Motschulsky (Geib et al., 2008) yielded a lignocellulose degrading member of the F. solani species complex (FSSC); phylogenetic species FSSC 6 and the xyleborine ambrosia beetle X. ferrugineus from Costa Rica was reportedly associated with Fusarium sp. AF-9 NRRL 22643 (Norris, 1980). Other fusaria have been reported from various other ambrosia beetles (Kolarik and Hulcr, 2008; Kostovcik et al., 2014), but their phylogenetic identity and ecological function remain unknown. In contrast to the Fusarium–Euwallacea mutualism, where the Fusarium symbiont is transmitted in mycangia and cultivated by females in galleries as a source of nutrition, A. glabripennis vertically transmits FSSC 6 in their gut (Geib et al., 2012), whereas various other fusaria associated with other clades of ambrosia beetles are mostly vectored phoretically (Bateman and Hulcr, unpublished).

In addition to sift-mating and haplodiploidy, the rapid radiation of the Xyleborini has been attributed to their specialization on ambrosia fungi that concentrate nutrients from living xylem tissue, thus allowing the beetles to greatly increase their range of woody hosts (Jordal et al., 2000). This is dramatically illustrated by the broad host range of the F. euwallacea – Euwallacea sp. #1 mutualists in southern California (Eskalen et al., 2013). While specificity to tree hosts is typically low among ambrosia beetles, specificity to their fungal symbionts may be high. Five different Euwallacea spp. analyzed here each cultivates a single AFC species. Only Euwallacea sp. #2 on avocado from Miami-Dade County, Florida and Euwallacea sp. #4 from Chinese tea in Sri Lanka cultivate two different fusaria, but this is still a relatively narrow range of symbionts compared to other ambrosia beetles, such as Xyleborus spp., where a single beetle can carry multiple symbiont genera (Carrillo et al., 2014; Kostovcik et al., 2014). Although preliminary data suggest that Euwallacea sp. #1 from Israel and California can complete its life cycle when grown on F. euwallaceae (AF-2), but not on F. ambrosium (AF-1) (Freeman et al., 2012), additional experiments are needed to determine whether this feed requirement is obligate. Preliminary data indicate that the culture medium may require optimization for each Euwallacea species (Cossé, unpublished).

There are several interesting parallels between the Euwallacea–Fusarium and other ambrosia beetle – fungus mutualisms, and the Macrotermitinae termites – Termotomycyces (Aanen et al., 2002) ectosymbioses. They all appear to have evolved monophyletically without any known reversals to a free living state, the fungal symbionts are transmitted vertically by one parent, host switching is common, and during each generation the larvae must acquire a suitable fungal symbiont (Nobre et al., 2011). Symbiont transmission in Euwallacea spp. #2 and #4 is mixed, in that fusaria are transmitted vertically in mycangia, but horizontal transfers via host-switching have occurred (Bright and Bulgheresi, 2010). With the exception of two early diverging taxa within AFC Clade A that are only known from dead wood from Sri Lanka (Kasson et al., 2013), which we speculate might be facultative associates of Euwallacea, the tight association of later diverging members of the AFC with mycangia of Euwallacea spp. or their galleries suggests that they may be obligate symbionts.

Interestingly, once Euwallacea galleries are abandoned they rapidly become overgrown by conidial fungi, including fast growing members of the F. solani species complex, which are otherwise common endophytes and saprophytes on living and dead woody plant hosts. Therefore, laboratory rearing experiments may help elucidate how the health of active fungus gardens is maintained. Research is also needed to ascertain whether Euwallacea spp., as reported for X. ferrugineus (Kok et al., 1970), can complete their life cycles on a fungus-free medium when fed sterols such as ergosterol isolated from the Fusarium they cultivate. The successful laboratory rearing of Euwallacea spp. #1 and #2 on artificial media (Carrillo and Cossé, unpublished) should facilitate experiments directed at elucidating basic aspects of their fungiculture, behavior and chemical ecology that are not feasible in their woody hosts (Biedermann et al., 2009).

4.2. Divergence-time estimates of the Euwallacea–Fusarium mutualism

Our analyses suggest a remarkable temporal correlation between the origin of the AFC-farming Euwallacea and the diversification of the AFC clade. Dated scolytine fossils (Jordal and Cognato, 2012) were used to date the evolutionary origin of the AFC-farming Euwallacea to the early Miocene 19.3 Mya [95% HPD interval: 10.7–28.2], which suggests the clade evolved early within the spectacular radiation of the Xyleborini ~21 Mya (Jordal and Cognato, 2012; Jordal et al., 2000). In the absence of a fossil record for Fusarium, five external calibration points were used to date the evolutionary origin of the AFC as previously described (Kasson et al., 2013; O’Donnell et al., 2013). Based on the coincident diversification-time estimates and their high endemism in southern Asia, the available data suggests the Euwallacea–Fusarium mutualism may have first evolved near the Oligocene–Miocene boundary ~19–24 Mya in tropical or subtropical Asia. The dated phylogeny indicates that the majority of cladogenic events within the AFC appear to have taken place relatively rapidly over the past 5 Ma, which helps explain the short internodes supporting the terminal taxa and poor support for the inferred cladogenic events within Clade B.

The Euwallacea–Fusarium symbiosis is relatively young compared to fungiculture in other ambrosia beetle groups that employ ophiostomoid and microascalean fungi, some of which date to more than 40 Mya (Jordal and Cognato, 2012). However, within Xyleborini, Euwallacea and its association with Fusarium appears to have evolved soon after the origin of the whole tribe approximately 20 Mya. It is likely that the xyleborine ancestors of Euwallacea that were already farming ophiostomatalean and microascalean fungi were associated with different fungal clades, and the AFC was adopted by Euwallacea de novo. This symbiosis is also younger than those of the fungus-farming termites (Nobre et al., 2011) and ant agriculture (Mueller et al., 1998; Schultz and Brady, 2008) that are estimated to have arisen approximately 31 and 50 Mya, respectively. Still, the divergence-time estimates should be viewed as relative chronological events and not as absolute dates, as evidenced by the broad confidence intervals on the dated phylogenies. This caveat is offered because dated phylogenies are well known to be subject to several sources of error, including rate heterogeneity among clades and uncertainty in the fossil ages used as calibration points (Berbee and Taylor, 2010; Taylor and Berbee, 2006).

4.3. Cophylogenetic analyses of the Euwallacea–Fusarium mutualism

In contrast to other methods for comparing phylogenies (Hughes et al., 2007), we elected to use the maximum parsimony...
event-based cophylogenetic tools Jane 4 (Conow et al., 2010) and CoRe-PA (Merkle et al., 2010) because they can accommodate multisymbiont hosts and molecular phylogenies that are not fully resolved. These tools use a single set of event costs that are either specified a priori by the user or automatically inferred based on a mathematical objective. Since tree reconciliations are known to be sensitive to the choice of event costs, we also used the costscape tool in the Xscape toolkit (http://www.cs.hmc.edu/~hadas/xscape/) to categorize and count all possible maximum parsimony reconciliations over a broad range of possible event costs (Libeskind-Hadas et al., 2014). Of the five groups of potentially optimal solutions that were found by costscape over a 25-fold range of possible event costs (cospeciation = 0, duplication normalized to 1, switch and loss both varying from 0.2 to 5), one group was found by both Jane 4 and CoRe-PA with 3 cospeciations, 0 duplications, 5 switches, and 0 losses. There were two nearly identical reconciliations with these event counts. Costscape also found a solution with 4 cospeciations, 0 duplications, 4 switches, and 1 loss that was not found by Jane nor CoRe-PA but this solution is optimal over a wide range of event costs. The remaining reconciliations found by costscape were only optimal for extreme values of the event costs and were thus unlikely to be plausible. We used the newly-developed sipscape tool in Xscape to analyze the statistical significance of the maximum parsimony reconciliations over the same broad range of event costs. This analysis failed to reject the null hypothesis that the Euwallacea–Fusarium trees might be similar by chance. Maximum parsimony-based reconciliation analyses of the Fusarium–Euwallacea mutualism conducted with Jane 4 and CoRe-PA suggests the incongruent phylogenies are best explained by multiple host shifts rather than diversifying coevolution (i.e., host-shift speciation sensu de Vienne et al., 2013). The little evidence for cospeciation inferred from the cophylogenetic analysis is not surprising, given that Euwallacea larvae, and those of other scolytine ambrosia beetles, must acquire fungal symbionts de novo each generation via horizontal transmission, thereby providing the opportunity for host switching (Six, 2012). Indeed, the relationship between many ambrosia beetles and their fungi is much more promiscuous than previously thought. Although some beetle groups have their preferred fungal symbionts (Harrington et al., 2014), sympatric beetles may also share a common pool of local ambrosia fungi (Carrillo et al., 2014; Kostovcik et al., 2014). Host-shift speciation appears to be the norm not just among beetles, but also in the broad spectrum of mutualistic associations between other organisms and fungi (reviewed in de Vienne et al., 2013), including the fungus-farming termites (Nobre et al., 2011) and Microbotryum anther smuts on caryophyllaceous hosts (Refrégier et al., 2008). The available data indicate that when host switching did occur in Euwallacea, Microbotryum and the fungus-growing termites, typically they were to closely related hosts. While the results of these and other studies indicate that long-term coevolution is rare on a macroevolutionary scale (de Vienne et al., 2013), the high symbiont fidelity of fungus-growing attine ants in the Cyphomyrmex wheeleri species group suggests that coevolving lineages can be maintained for several million years (Meidibadi et al., 2012).

Results of the present study highlight the importance of resolving species limits of invasive pests and pathogens of quarantine importance. Our GCPSR-based analyses indicate that five different exotic Euwallacea spp. cultivating six different members of the AFC have been introduced into the United States. With the exception of E. validus, first detected on Long Island, New York in 1976 (Rabaglia et al., 2006), the other four Euwallacea spp. appear to have been introduced just within the past one to two decades. The present results should help inform quarantine officials and agricultural scientists of each species’ genetic diversity, host range and geographic distribution so that they can be monitored using molecular markers such as those developed in this and our previous study (Kasson et al., 2013). While our results indicate that some Euwallacea spp. appear to have switched AFC symbionts in Asia, it remains to be determined whether these invasive beetles can complete their life cycle cultivating newly acquired ambrosia fungi, including other members of the AFC. At least two pairs of exotic Euwallacea spp. are already sympatric within the United States: Euwallacea sp. #2 and E. interjectus are increasingly common throughout Florida (K. Okins, Cooperative Agricultural Pest Survey, Florida Department of Agriculture and Consumer Services, pers. comm.) and E. validus and E. interjectus have been trapped in northern Georgia and other locations throughout the southeastern United States (Atkinson, 2014). Now that they are sympatric, the chance that they might cohabit common hosts and exchange AFC symbionts is theoretically possible. It is worth noting that the exotic wood-inhabiting wasp Sirex switched Amylostereum mutualists after introduction into Canada (Wooding et al., 2013). Also, six native or previously established species of ambrosia beetles began transmitting Raffaelea lauricola T.C. Harr., Fraedrich and Aghayeva, the wilt pathogen of redbay (Persea borbonia (L.) Spreng.), after it was introduced into the United States from Asia (Carrillo et al., 2014). Similarly, the seed bug Orsillus maculatus Fieber began vectoring Seiridium cardiale (Wagener) Sutton after this aggressive cypress canker pathogen was introduced into Europe (Battisti et al., 1999). These and other examples serve to illustrate that novel pest–pathogen associations can pose significant challenges to control and quarantine efforts (Wingfield et al., 2010). Lastly, the host switching that has helped shaped the Fusarium–Euwallacea mutualism highlights the real possibility that more virulent and aggressive combinations of these economically destructive mutualists could evolve over time.

Acknowledgments

The authors thank Nathane Orwig for running sequences in the NCAUR DNA Core Facility, Thomas White and Ronald Wideman for technical assistance in Homestead, CABI Biosciences for providing Fusarium isolates, Paul R. Campbell and Andrew D.W. Geering, The University of Queensland, Australia for collections of Euwallacea sp. #3, and Pat Nolan, County of San Diego, California for collections of Euwallacea sp. #5. The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture or other firms or similar products not mentioned. The USDA is an equal opportunity provider and employer. JH and CB were funded by the USDA Forest Service and the National Science Foundation, MK was funded by the USDA Forest Service’s Forest Health Technology Enterprise Team, and AE and FN were supported by the USDA Forest Service and the California Avocado Commission.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2014.10.014.

References


