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

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ABSTRACT

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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 fungiculture 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 Ambrosia *Fusarium* Clade (ACF) 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 *F. 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 cospeciation 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 *E. 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.

Additional keywords: cophylogeny, fungiculture, hybrid introgression, molecular phylogenetics, phylogenetic species, symbiosis

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 microascalean and ophiostomatalean fungi. The fusaria associated with *Euwallacea* form a monophyletic group (Ambrosia *Fusarium* Clade, or AFC) within the *F. 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 (Carrillo et al., 2012; Eskalen and Stouthamer, 2012; Haack, 2006; Kasson et al., 2013; Kirkendall and Ødegaard, 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* (Gadd & Loos) Agnihothr. & 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 *F. euwallaceae* S. Freeman et al. (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://eskalenlab.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 mannoheptulose 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: *E. validus* Eichhoff and *E. interjectus* Blandford. *Euwallacea validus* is a temperate species with broad tree host range (Rabaglia 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). *Euwallacea 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* spp. 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 TSHB.

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 (Gadd and Loos, 1947; Nirenberg, 1990). Based on morphological differences from *F. ambrosium* and phylogenetics, *F. euwallaceae* was described for AF-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 cophylogenetic 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 sec 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 (½ PDA+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://nrml.ncaur.usda.gov/TheCollection/Requests.html>). Other fungal symbionts were recovered from *Euwallacea*, including *Raffaelea* 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 Xyleborini 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. fornicatus*, *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-1 α* , 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, 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-1 α* (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 largest (*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 $\geq 70\%$, 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) with PAUP* 4.0b10, employing 1000 pseudoreplicates of the data, 10 random taxon-addition sequences per replicate, TBR branch swapping, with MAXTREES set to automatically increase by 100. Maximum likelihood (ML) analyses were conducted with GARLI ver. 1.0 (ML, Zwickl, 2006) on the CIPRES Science Gateway site (<http://www.phylo.org>) using the default GTR+ Γ + I model of molecular evolution. ML bootstrap support was assessed by 1000 pseudoreplicates of the data.

BEAST ver. 2.1.3 (Bouckaert et al., 2014) was used to generate divergence-time estimates for *Euwallacea* and *Fusarium* as described in O'Donnell et al. (2013). Published stem and crown ages of the Xyleborini (Jordal and Cognato, 2012) and dates for five 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 birth-death (Gernhard, 2008) and calibrated Yule models as the tree priors. An uncorrelated lognormal relaxed molecular clock (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 95% highest probability density (HPD) intervals.

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: cospeciation = 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 linked by the auxiliary lines in Fig. 1. Because predefined event costs are difficult to predict a priori, the *costscape* tool in the Xscape cophylogeny software tool set (Libeskind-

Hadas et al., 2014; <http://www.cs.hmc.edu/~hadas/xscape/>) was used to compute the set of Pareto-optimal event count vectors and visualize the costs of host switch or loss over the range of 0.2 to 5 in the *Euwallacea* – *Fusarium* dataset. The algorithmic approach used in the *costscape* tool in Xscape uses a reconciliation algorithm that can in theory result in temporal inconsistencies. By contrast, the one used in Jane 4 does not permit such inconsistencies, but it also does not guarantee that all optimal solutions will be found. Empirical p-values were computed using the *sigscape* tool in Xscape for host switch and loss costs ranging from 1 to 5 based on 1000 permutations.

3. Results

3.1. Phylogenetic diversity of the *Fusarium* – *Euwallacea* mutualism

By DNA typing *Fusarium*, and the *Euwallacea* from which they were isolated, we were able to obtain a preliminary estimate of symbiont fidelity, as reflected by the color-coded auxiliary lines used to link nine fusaria with the seven *Euwallacea* spp. that cultivate them (Fig. 1). For the AFC fusaria, we continue to follow the previously developed notation for species lineages (Kasson et al., 2013), which now number twelve (AF 1-12), with AF-1 and AF-2 representing *F. ambrosium* and *F. euwallaceae*, respectively (Fig. 1). MP phylogenetic analysis of a 25-taxon, 4-gene dataset (*EF-1 α* , 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 *F. neocosmosporiellum* O'Donnell & Geiser (formerly *Neocosmospora vasinfecta* E.F. Sm.) as the outgroup based on more inclusive analyses of the *F.*

solani species complex (O'Donnell et al., 2008). While *RPB1* (159 PIC) and *RPB2* (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 $\geq 70\%$ 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 *F. sp.* AF-10 from Singapore could not be tested because it was represented by a single, genetically divergent isolate, GCPSR-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, 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).

Maximum likelihood and maximum parsimony analyses of a 23-taxon, 6-gene *Euwallacea* dataset (*EF-1 α* , CAD, ArgK, 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 distinct singleton 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-1 α* with 17 PIC contributed the least. Although the monophyly of a single collection from Papua New Guinea designated as *Euwallacea* sp. #6 *E. 'fornicatus'* (Cognato et al., 2011) could not be tested, the other seven *Euwallacea* lineages were supported as genealogically exclusive by GCPSR-based analyses of the individual and combined dataset (Fig. 1, right). With the exception of the 373 bp *EF-1 α* 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 $\geq 70\%$ (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).

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-1 α* 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 (Bouckaert 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 radiation 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 cospeciations 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 $\langle c, d, s, l \rangle$ indicating the number of cospeciations, duplications, host switches, and losses, respectively. One group of solutions had event count vector $\langle 3, 0, 5, 0 \rangle$ 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 $\langle 4, 0, 4, 1 \rangle$. Three other groups of solutions had event count vectors of $\langle 4, 2, 2, 1 \rangle$, $\langle 3, 4, 1, 20 \rangle$, and $\langle 3, 5, 0, 27 \rangle$. 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 *sigscape* tool in Xscape. When host switch and loss costs ranging from 1 to 5 were analyzed, 100% of the event cost space found by *sigscape* was not significant at the 0.05 level (data not shown). Thus, the null hypothesis that the three cospeciations 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

439 study and discovered a similar pattern of apparent cryptic speciation among the ambrosia
 440 beetles that farm them. In *Fusarium*, we have now detected 11 species according to the criteria
 441 of genealogical concordance and non-discordance (GCPSR, Taylor et al. 2000; Dettman et al.,
 442 2003). These 11 reciprocally monophyletic species lineages were supported by bootstrap
 443 analyses of one or more of the single-gene genealogies and the combined dataset. Within the
 444 sampled *Euwallacea*, we detected seven phylogenetically distinct species lineages using the
 445 same criteria, five of them within the morphological concept of *E. fornicatus*. Although the
 446 taxonomic status of the single isolate of *F. sp.* AF-5 recovered from a beetle-infested rubber
 447 tree (*Hevea brasiliensis* Müll. Arg.) in Malaysia and an ambrosia beetle identified as *E.*
 448 *fornicatus* from Papua New Guinea (Cognato et al., 2011) could not be evaluated employing
 449 GCPSR-based criteria, they very likely represent phylogenetically distinct species based on the
 450 level of genetic divergence from their sister clades. Two additional findings support our
 451 interpretation of species diversity within the AFC and the *Euwallacea* spp. that cultivate them
 452 (i) unique AFC symbionts are cultivated by each of the seven *Euwallacea* lineages, suggesting
 453 symbiosis-related adaptations towards maintaining vertical transmission, and (ii) the
 454 divergence-time estimates indicate the youngest species pairs we sampled within *Fusarium* and
 455 *Euwallacea* diverged ~0.6 and 1.5 Mya, respectively. The three fusaria discovered herein
 456 produced distinctive club-shaped macroconidia similar to those produced by six other members
 457 of the AFC (see Fig. 3 in Kasson et al., 2013). We posit that these spores represent a symbiosis-
 458 related adaptation, analogous to nodules, gongylidia or swollen cells produced by fungi that are
 459 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 nine 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 catalogue indicates was isolated from *Xyleborus ferrugineus* 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 et al., 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. commun.). An isolate of AF-9 (Table 2) was also recovered in the present study from beetle-infested royal poinciana, *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 (*Hevea brasiliensis*) in Malaysia and AF-10 received as IMI 351954 *F. 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 crypsis observed here for *Euwallacea* is consistent with that reported for numerous other Xyleborini, 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. *pisi* W.C.

Snyder & 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* into the population of *Euwallacea* sp. #2 in Miami-Dade County, Florida based on analyses of the individual nuclear and mitochondrial gene partitions. The xenologous allele was preferentially amplified with conserved CO1 primers (Dole et al., 2010), whereas the orthologous copy was only detected using *Euwallacea* sp. #2-specific CO1 primers developed in the present study (Supplementary Table S3). If these two species did hybridize, it probably took place in Asia because they do not appear to be sympatric in North America. Moreover, the hybrid introgression is assumed to have taken place relatively recently given that the *Euwallacea* sp. #2 CO1 xenolog and *E. validus* ortholog are identical (Fig. 2). It is worth mentioning that the haploid, blind and flightless males occasionally leave their galleries and wander around trees in search of other galleries, thus providing the opportunity for interspecific matings (Hulcr, unpubl.). We discounted incomplete lineage sorting because (i) *E. validus* and *Euwallacea* sp. #2 are distantly related, having shared a most recent common ancestor ~12.4 Mya (Fig. 1), and (ii) in contrast to the CO1 phylogeny (Fig. 2), the four nuclear genes and mitochondrial 16S rDNA support the monophyly of these two species. Interspecific introgression as reported here is another potential pitfall of taxon identification using a single gene such as CO1 (Rubinoff et al., 2006). It is also worth noting that DNA sequence data from this gene cannot be used for the reliable identification of *Fusarium* due to the presence multiple paralogs in a number of the species (Gilmore et al., 2009).

Because paternal inheritance of mitochondria is relatively unusual (Dowling and Secor, 1997), particularly in the case of inbreeding species, special steps were taken to verify our findings of hybrid introgression. First, the thorax+abdomen of the two putative *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 Bertheau 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., 2006; 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 xyleborine 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* (Hurst and Jiggins, 2004).

Of the 11 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 xyleborine genus *Euwallacea*. All other known ambrosia beetles engage in mutualism with symbionts that belong to other fungal genera, mostly from Ophiostomatales and Microascales (Massoumi Alamouti 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 Xyleborini, 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* (Ophiostomatales) 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* fungiculture was the tea shot hole borer (TSHB), *E. fornicatus* (formerly known as *Xyleborus fornicatus*), which cultivates *F. ambrosium* in Chinese tea in Sri Lanka and India (Brayford 1987; Gadd and Loos, 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 *Xyleborus 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 (C. Bateman and J. Hulcr, unpubl.).

In addition to sib-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. euwallaceae* – *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., 2013; 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, unpubl.).

There are several interesting parallels between the *Euwallacea* – *Fusarium* and other ambrosia beetle – fungus mutualisms, and the Macrotermitinae termites – *Termitomyces* (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, unpubl.) 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 cladogenetic events within the AFC appear to have taken place relatively rapidly over the past 5 My, which helps explain the short internodes supporting the terminal taxa and poor support for the inferred cladogenetic 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, 2013). 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 *sigscape* 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., 2013; 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 cospeciation 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 (Mehdiabadi 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 & 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 cardinale* (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.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/>.

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990

991 FIGURE LEGENDS

992

993 Fig. 1. (Left) One of nine most-parsimonious trees (MPTs) inferred from the combined four-
 994 gene data set (*EF-1 α* , ITS+28S rDNA, *RPB1* and *RPB2*) for 12 Ambrosia *Fusarium* Clade
 995 (identified by red internode) species [AF-1 through AF-12]. Thickened black internodes identify
 996 Clades A and B within the Ambrosia *Fusarium* Clade. Color-coding of auxiliary lines are used to
 997 link nine fusaria with the *Euwallacea* ambrosia beetles that cultivate them. The putative
 998 fungus-farming xyleborine associated with AF-5 from Malaysia, AF-9 from Miami-Dade County,
 999 Florida and AF-10 from Singapore is unknown. (Right) Maximum parsimony analysis of a six-
 1000 gene data set (*ArgK*, *CAD*, *CO1*, *EF-1 α* , 16S rDNA and 28S rDNA) resolved seven fungus-farming
 1001 *Euwallacea* spp. Two *Euwallacea* spp., *Euwallacea* sp. #2 infesting avocado in Miami-Dade
 1002 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 $\geq 70\%$, 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 $\leq 5\%$ of the MP-BS value. The *Fusarium* and *Euwallacea* phylograms were rooted on sequences of *F. neocosmosporiellum* (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. 1 and 2. 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.

Fig. 2. Opposed maximum parsimony (MP) phylograms inferred from the nuclear large subunit 28S rDNA D2/D3 regions (left) and mitochondrial cytochrome oxidase subunit 1 (CO1) gene (right) from five *Euwallacea* spp., three of which are listed as *Euwallacea* sp. #1-3. Species are distinguished using the same color codes as in Fig. 1. *Euwallacea* sp. #2 is resolved as a genealogically exclusive lineage in the 28S rDNA phylogeny, but it is paraphyletic in the CO1 gene tree. In addition to possessing an orthologous CO1 allele, two *Euwallacea* sp. #2

collections from avocado in Miami-Dade County, Florida (RP-30 and RP-31 in bold black font) share an identical putatively xenologous allele with *E. validus*. The most plausible explanation is hybrid introgression between *E. validus* and *Euwallacea* sp. #2. Because these exotic xyleborines are not known to be sympatric in North America, we speculate that the interspecific hybridization event took place in Asia where they are endemic. The phylograms were rooted on sequences of *Wallacellus similis* from Papua New Guinea (Cognato et al., 2011). Numbers above internodes represent MP bootstrap support based on 1000 pseudoreplicates of the data. CI, consistency index; MPTs, most-parsimonious trees; PIC, parsimony informative characters; RI, retention index.

Fig. 3. Cophylogenetic analysis of the *Euwallacea* – *Fusarium* mutualism conducted with Jane 4 (Conow et al., 2010) using the following event costs: cospeciation = 0, duplication = 1, host switch = 1, lineage sorting = 1 and failure to diverge = 1. The best-fit reconciliation of the *Euwallacea* and *Fusarium* trees included five host shifts (i.e., switches to different fusarial symbionts) and three cospeciations. The *Fusarium* and *Euwallacea* phylogenies were inferred from sequences of one representative of the nine fusaria and *Euwallacea* linked by the color-coded auxiliary lines in Fig. 1. The number by each co-divergence or duplication event represents the percentage of solutions in which the *Euwallacea* tree was mapped to this location on the *Fusarium* tree.

Fig. 4. Pareto-regions computed by the *costscape* tool in Xscape (Libeskind-Hadas et al., 2014) for the 9-taxon *Fusarium* and *Euwallacea* datasets employed in the Jane 4 cophylogenetic

analysis reported in Fig. 3. The legend identifies four event counts (i.e., cospeciation = 0, duplication = 1, host-switch = 1 and lineage sorting = 1) for each of the five color-coded regions. The 'count' field for each region indicates the number of unique reconciliations found by *costscape*. The two <3,0,5,0> reconciliations were deemed the most-parsimonious solutions by CoRe-PA (Merkle et al., 2010); Jane 4 found one of these (Conow et al., 2010). The two optimal reconciliations differed only as to whether one of the host shifts was initially to AF-11 followed by one to *F. ambrosium* (AF-1), as illustrated in Fig. 3, or the other way around. Note that the third and fourth regions in the legend occupy zero (i.e., <4,2,2,1>) or nearly zero area (i.e., <3,4,1,20>) and are barely visible in the plot.

Supplementary Fig. S1. Maximum clade credibility (MCC) tree showing divergence time estimates for the Ambrosia Fusarium Clade (AFC) obtained using BEAST ver. 2.1.3 (Bouckaert et al., 2014). The MCC tree was generated using an uncorrected lognormal relaxed molecular clock with a birth-death model as the tree prior. A priori divergence dates for the BEAST analysis were based on those published for five ascomycetous fungi (Kasson et al., 2013). Numbers at each internode indicate median age together with the Bayesian 95% highest probability density interval in brackets. Geological time is in millions of years before present (Mya). Oligo = Oligocene, Pleis = Pleistocene.

Supplementary Fig. S2. Maximum clade credibility (MCC) tree showing divergence time estimates for the AFC-farming *Euwallacea* obtained with BEAST ver. 2.1.3 (Bouckaert et al., 2014). An uncorrected lognormal relaxed molecular clock with a birth-death model as the tree

1069 prior was used to generate the MCC tree. A priori divergence dates for the BEAST analysis were
1070 obtained from crown ages published for the Xyleborini (Jordal and Cognato, 2012). Sequences
1071 of four closely related taxa in the Scolytinae (i.e., *Cnestus bimaçulatus* Eggers, *Anisandrus dispar*
1072 Eichhoff, *Ozopemon uniseriatus* Eggers and *Dryocoetes autographus* Ratzenburg, were
1073 downloaded from GenBank. Median age and the Bayesian 95% highest probability density
1074 interval in brackets is indicated at each node. Geological time is in millions of years before
1075 present (Mya). Pleis = Pleistocene.

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Table 1. Histories of xyleborines included in the phylogenetic analyses (Figs. 1 and 2).

Sequence ID ^a	Collection # ^b	Host/Substrate	Geographic Origin
<i>Euwallacea 'forficatus'</i>	Zool. Scripta 40:174–186 (2010)	<i>Artocarpus communis</i> (breadfruit)	Papua New Guinea
<i>Euwallacea destruens</i>	Zool. Scripta 40:174–186 (2010)	<i>Pterocymbium beccarii</i> (amberoi)	Papua New Guinea
<i>Euwallacea interjectus</i>	CB-8965	<i>Acer negundo</i> (box elder)	Gainesville-FL
<i>Euwallacea interjectus</i>	CB-4024	<i>Acer negundo</i> (box elder)	Gainesville-FL
<i>Euwallacea interjectus</i>	CB-4025	<i>Acer negundo</i> (box elder)	Gainesville-FL
<i>Euwallacea validus</i>	MK-MD1-1	<i>Ailanthus altissima</i> (tree-of-heaven)	MD-USA
<i>Euwallacea validus</i>	MK-P1	<i>Ailanthus altissima</i> (tree-of-heaven)	OH-USA
<i>Euwallacea validus</i>	MK-C1	<i>Ailanthus altissima</i> (tree-of-heaven)	PA-USA
<i>Euwallacea</i> sp. #1	SF-1	<i>Persea americana</i> (avocado)	Israel
<i>Euwallacea</i> sp. #1	SF-M1	<i>Persea americana</i> (avocado)	Israel
<i>Euwallacea</i> sp. #1	AE-55	<i>Quercus robur</i> (English oak)	Los Angeles-CA
<i>Euwallacea</i> sp. #1	AC-1	<i>Acer negundo</i> (box elder)	Los Angeles-CA
<i>Euwallacea</i> sp. #2	JK-35	<i>Persea americana</i> (avocado)	Miami-Dade Co.-FL
<i>Euwallacea</i> sp. #2	JK-37	<i>Persea americana</i> (avocado)	Miami-Dade Co.-FL
<i>Euwallacea</i> sp. #2	DC-2	<i>Persea americana</i> (avocado)	Miami-Dade Co.-FL
<i>Euwallacea</i> sp. #2	RP-29	<i>Persea americana</i> (avocado)	Miami-Dade Co.-FL
<i>E. validus</i> - <i>Euwallacea</i>	RP-30	<i>Persea americana</i> (avocado)	Miami-Dade Co.-FL
<i>E. validus</i> - <i>Euwallacea</i>	RP-31	<i>Persea americana</i> (avocado)	Miami-Dade Co.-FL
<i>Euwallacea</i> sp. #3	AG-1	<i>Persea americana</i> (avocado)	Queensland, Australia
<i>Euwallacea</i> sp. #3	AG-2	<i>Persea americana</i> (avocado)	Queensland, Australia
<i>Euwallacea</i> sp. #4	PL-KT2	<i>Camellia sinensis</i> (tea)	Kandy, Sri Lanka
<i>Euwallacea</i> sp. #4	PL-KT-10	<i>Camellia sinensis</i> (tea)	Kandy, Sri Lanka
<i>Euwallacea</i> sp. #4	PL-TT1	<i>Camellia sinensis</i> (tea)	Talawakelle, Sri Lanka
<i>Euwallacea</i> sp. #5	PN-RW1	<i>Salix laevigata</i> (red willow)	San Diego Co.-CA
<i>Euwallacea</i> sp. #5	PN-SY1	<i>Platanus racemosa</i> (California sycamore)	San Diego Co.-CA
<i>Wallacellus similis</i>	Zool. Scripta 40:174–186 (2010)	<i>Pterocymbium beccarii</i> (amberoi)	Papua New Guinea

^a The dataset consisted of portions of the following six genes: *argK*, *EF-1 α* , *CAD*, *cox1*, 16S rDNA and 28S rDNA. *Euwallacea 'forficatus'* w

^b Sequences of three xyleborines from Papua New Guinea reported on in Zool. Scripta 40:174-186(2010) were downloaded from GenBank

Table 2. Histories of Ambrosia Fusarium Clade isolates included in the phylogenetic analysis (Fig. 1, left).

Sequence ID ^a	NRRL and equivalent # ^b	Xyleborine associate ^c	Host/Substrate	Geographic Origin			
AF-1 <i>Fusarium ambrosium</i>	NRRL 20438 = IMI 296597	<i>Euwallacea 'forficatus'</i>	<i>Camellia sinensis</i> (tea)	India			
AF-1 <i>Fusarium ambrosium</i>	NRRL 62942 = KOD 799 = PL-KH10	<i>Euwallacea</i> sp. #4	<i>Camellia sinensis</i> (tea)	Sri Lanka			
AF-2 <i>Fusarium euwallaceae</i>	NRRL 54727 = CBS 135859	<i>Euwallacea</i> sp. #1	<i>Persea americana</i> (avocado)	Israel			
AF-2 <i>Fusarium euwallaceae</i>	NRRL 62626 = AE-1854	<i>Euwallacea</i> sp. #1	<i>Acer negundo</i> (box elder)	Los Angeles, CA			
AF-3 <i>Fusarium</i> sp.	NRRL 62606 = CB-1499	<i>Euwallacea interjectus</i>	<i>Acer negundo</i> (box elder)	Gainesville-FL			
AF-3 <i>Fusarium</i> sp.	NRRL 62629 = CB-1191	<i>Euwallacea interjectus</i>	<i>Acer negundo</i> (box elder)	Gainesville-FL			
AF-4 <i>Fusarium</i> sp.	NRRL 62578 = FRC S-2576	<i>Euwallacea validus</i>	<i>Ailanthus altissima</i> (tree-of-heaven)	PA-USA			
AF-4 <i>Fusarium</i> sp.	NRRL 62579 = FRC S-2581	<i>Euwallacea validus</i>	<i>Ailanthus altissima</i> (tree-of-heaven)	PA-USA			
AF-5 <i>Fusarium</i> sp.	NRRL 22231 = IMI 110107	unknown	<i>Hevea brasiliensis</i> (rubber tree)	Malaysia			
AF-5 <i>Fusarium</i> sp.	NRRL 46518 = FRC S-2075	unknown	<i>Hevea brasiliensis</i> (rubber tree)	Malaysia			
AF-6 <i>Fusarium</i> sp.	NRRL 62590 = RP-AF9	<i>Euwallacea</i> sp. #2	<i>Persea americana</i> (avocado)	Miami, FL			
AF-6 <i>Fusarium</i> sp.	NRRL 62591 = RP-16-1B	<i>Euwallacea</i> sp. #2	<i>Persea americana</i> (avocado)	Miami, FL			
AF-7 <i>Fusarium</i> sp.	NRRL 62610 = AG-1	<i>Euwallacea</i> sp. #3	<i>Persea americana</i> (avocado)	Queensland, Australia			
AF-7 <i>Fusarium</i> sp.	NRRL 62611 = AG-2	<i>Euwallacea</i> sp. #3	<i>Persea americana</i> (avocado)	Queensland, Australia			
AF-8 <i>Fusarium</i> sp.	NRRL 62584 = RP-Amb2	<i>Euwallacea</i> sp. #2	<i>Persea americana</i> (avocado)	Miami, FL			
AF-8 <i>Fusarium</i> sp.	NRRL 62585 = RP-AF4	<i>Euwallacea</i> sp. #2	<i>Persea americana</i> (avocado)	Miami, FL			
AF-9 <i>Fusarium</i> sp.	NRRL 22643 = ATCC 44215	<i>Xyleborus ferrugineus</i>	na	Costa Rica			
AF-9 <i>Fusarium</i> sp.	NRRL 66088 = KOD 838	unknown	<i>Delonix regia</i> (royal poinciana)	Miami, FL			
AF-10 <i>Fusarium</i> sp.	NRRL 62941 = KOD 147 = IMI 351954	unknown	unknown	Singapore, Malaysia			
AF-11 <i>Fusarium</i> sp.	NRRL 62943 = KOD 796 = PL-KH1	<i>Euwallacea</i> sp. #4	<i>Camellia sinensis</i> (tea)	Sri Lanka			
AF-11 <i>Fusarium</i> sp.	NRRL 62944 = KOD 797 = PL-KH2	<i>Euwallacea</i> sp. #4	<i>Camellia sinensis</i> (tea)	Sri Lanka			
AF-12 <i>Fusarium</i> sp.	NRRL 62945 = KOD 793 = AE-FD420-G47	<i>Euwallacea</i> sp. #5	<i>Platanus racemosa</i> (California sycamore)	San Diego, CA			
AF-12 <i>Fusarium</i> sp.	NRRL 62946 = KOD 795 = AE-FD422-G69	<i>Euwallacea</i> sp. #5	<i>Platanus racemosa</i> (California sycamore)	San Diego, CA			
<i>Fusarium neocosmosporiellum</i>	NRRL 22468 = CBS 562.70	na	Stored peanuts	Guinea			
<i>Fusarium neocosmosporiellum</i>	NRRL 43467 = CBS 139182	na	Human eye	LA-USA			
^a Genealogical concordance phylogenetic species recognition (Taylor et al., 2000) revealed that partial <i>EF-1 α</i> can resolve all of the fusaria included in the present study except for AF-4 and AF-12.							

Table 3. Statistics for the individual and combined *Fusarium* partitions.

Locus	# bp	# MPTs ^a	MPT Length	CI ^b	RI ^c	UIC ^d	PIC ^e	# Nodes/# species receiving ≥70% MP/ML-BS ^f
<i>EF-1α</i>	678	2	73	0.82	0.91	7	50	8/6
ITS+LSU rDNA	1004	36	38	0.89	0.93	8	24	4/3
<i>RPB1</i>	1588	4	200	0.90	0.94	10	159	11/7
<i>RPB2</i>	1635	366	227	0.74	0.85	8	155	10/9
combined	4914	9	566	0.78	0.87	33	388	16/11

^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.

Locus	# bp	# MPTs ^a	MPT Length	CI ^b	RI ^c	UIC ^d	PIC ^e	# Nodes/# species receiving ≥70% MP/ML-BS ^f
ArgK	669	>50,000	82	0.89	0.94	23	46	5/2
CAD	469	>50,000	64	0.89	0.93	20	35	3/2
<i>EF-1α</i>	373	>50,000	37	0.89	0.92	16	17	1/0
LSU rDNA	1004	234	282	0.93	0.97	50	154	8/2
CO1	689	15,702	382	0.65	0.85	13	187	7/7
16S rDNA	420	>50,000	93	0.85	0.93	2	60	4/4
combined	3624	180	951	0.79	0.90	124	499	11/7

^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.

Figure 1

Figure 2

Figure 3

Figure 4