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COEVOLUTION AND COSPECIATION IN A BARK-BEETLE FUNGAL SYMBIOSIS

By

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Dissertation

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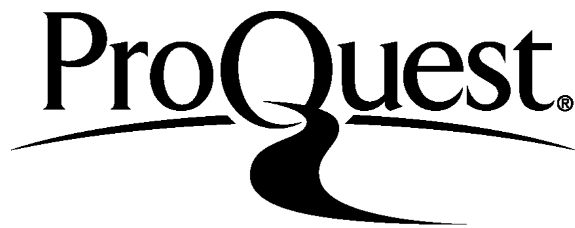

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Coevolution and Cospeciation in a bark-beetle fungal symbiosis

Chairperson: Diana L. Six

Bark beetles in the genus *Dendroctonus* are some of the most important insects in forest ecosystems worldwide and are known to be involved in symbiotic relationships with fungi. However, we have a poor understanding of beetle-fungal coevolution and if the specificity we see in beetle-fungal relationships may translate to cospeciation over longer time frames. In this dissertation, I attempt to answer these questions by investigating the western pine beetle (*Dendroctonus brevicomis*)-fungal symbiosis, a beetle that consists of two putative cryptic species in the early stages of divergence on two susbspecies of ponderosa pine (*Pinus ponderosa*). In the first chapter, I describe research aimed at determining if the western pine beetle harbors the same species of mycangial fungi across its entire range. I found widespread fungal fidelity and that the beetle carries two species, *Entomocorticium* sp. B, and *Ceratocystiopsis brevicomi*. In the second chapter, I describe research aimed at determining if the western pine beetle is indeed in an obligate mutualism and is adapted to fungal isolates with a shared evolutionary history. I found that *E*. sp. B was crucial for the successful development of western pine beetles and found no significant difference in the effects of the natal (shared history) and non-natal (no shared history) isolate on beetle fitness parameters. However, brood adult beetles failed to incorporate the non-natal fungus into their mycangium indicating adaption by the beetle to particular genotypes of symbiotic fungi. In the third chapter I describe research exploring if the beetle, its two fungal symbionts, and the host tree are genetically structured in a similar fashion indicative of shared evolutionary history and cospeciation. I sequenced the genomes of the beetle and two fungal symbionts and conducted population genetic and genomic analyses for the host tree, beetle, and for the two fungal symbionts. I found congruent patterns of population genetic structure and phylogenetic relationships between multiple species. Taken together, my dissertation research suggests that the western pine beetle is in a tightly linked and evolving obligate mutualism with fungi and that the entire tree-beetle-fungi system is diverging and cospeciating in concert.

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DISSERTATION OVERVIEW

The research contained herein was conducted in partial fulfillment of the Ph.D. requirements at the University of Montana. The dissertation contains three chapters which were each prepared as separate papers for publication. The first two chapters have been published and the necessary forms and/or citations for their reprint are included with these chapters. Each chapter contains the relevant review and background for the study. What follows is a brief description of each of the chapters in the dissertation and their relevance.

CHAPTER 1. Broadscale specificity in a bark beetle-fungal symbiosis: A spatio-temporal analysis of the mycangial fungi of the western pine beetle

In chapter 1 I describe research that was conducted to identify the fungal partners in the western pine beetle (*Dendroctonus brevicomis*) system and determine the level of fungal fidelity across the geographic range of the insect. I found that the beetle carries *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi* across its entire range. Further, through repeated sampling at two sites in Montana, I identified that *E*. sp. B was more prevalent throughout the beetle's flight season and that beetles captured with *E*. sp. B were on average larger than those captured with *C. brevicomi*. In total, the results show that the beetle shows widespread fungal fidelity and that one fungal symbiont, *E*. sp. B may provide more benefit to the beetle. This is the first paper comprehensively surveying the mycangial fungi of the western pine beetle and establishes the remarkable fungal specificity in the system.

CHAPTER 2. Experimental evidence of bark beetle adaptation to a fungal symbiont

In chapter 2 I describe the results of experimentally manipulating the beetle-fungal symbiosis. Given results from chapter 1 which suggested that *E.* sp. B was geographically widespread, more prevalent, and may provide more benefits to the beetle, I aimed to test if the beetle-fungal symbiosis is an obligate mutualism. Further, given that the western pine beetle consists of two putative cryptic species and results from chapter 1 suggested genetic differentiation in *E.* sp. B consistent with cryptic beetle species boundaries, I tested if beetles were adapted to particular fungal symbionts that share an evolutionary history. Results indicated that the beetle is indeed highly dependent on fungi and I observed a near complete loss of brood production in beetles reared without their fungal symbiont. Interestingly, beetles reared with an *E.* sp. B isolate originating from the other cryptic beetle species did not alter numbers of brood produced and the brood were similar in size and development time. However, those brood adults failed to incorporate the fungal isolate from the other cryptic beetle species into their mycangia. This is the first paper to experimentally demonstrate bark beetle adaptation to a fungal symbiont and suggest that mycangia may be fine-tuned to promote particular fungal associations.

CHAPTER 3. Cascading speciation among mutualists and antagonists in a tree-beetle-fungal interaction

In chapter 3 I describe the results of population genetic and genomic analyses of ponderosa pine, western pine beetle, and the two fungal symbionts. I conducted analyses of the four species to help determine if all species show patterns of genetic codifferentiation and phylogenetic patterns indicative of cospeciation. To characterize genetic structure and variation and infer the evolutionary history of these interacting species I first sequenced and assembled the

genomes of the western pine beetle, *C. brevicomi* and, *E.* sp. B. I then used reduced representation sequencing (RADseq) of multiple individuals of the beetle and fungi coupled with previously published data for the tree to conduct an integrated analyses of the four species. I found strong evidence that the host tree, the western pine beetle, and its two fungal symbionts show remarkably consistent patterns of population structure and codifferentiation. Further, three species of *E.* sp. B appear to be present in the symbiosis and these three species have dramatically reduced genetic variation yet are highly differentiated from one another. This is the first paper to demonstrate a 'phylogenetic cascade' of codifferentiation in a system outside of an insect-parasitoid system and suggests that coevolution coupled with geographic isolation may promote diversification and speciation in mutualisms as well.

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CHAPTER 1

Broadscale specificity in a bark beetle-fungal symbiosis: A spatio-temporal analysis of the mycangial fungi of the western pine beetle

Bracewell, R.R. and D.L. Six, *Broadscale Specificity in a Bark Beetle-Fungal Symbiosis: a Spatio-temporal Analysis of the Mycangial Fungi of the Western Pine Beetle.* Microbial Ecology, 2014. **68**(4): p. 859-870

ABSTRACT

Whether and how mutualisms are maintained through ecological and evolutionary time is a seldom studied aspect of bark beetle-fungal symbioses. All bark beetles are associated with fungi and some species have evolved structures for transporting their symbiotic partners. However, the fungal assemblages and specificity in these symbioses are not well known. To determine the distribution of fungi associated with the mycangia of the western pine beetle (*Dendroctonus brevicomis*), we collected beetles from across the insect's geographic range including multiple genetically distinct populations. Two fungi, *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi*, were isolated from the mycangia of beetles from all locations. Repeated sampling at two sites in Montana found that *E.* sp. B was the most prevalent fungus throughout the beetle's flight season, and that females carrying that fungus were on average larger than females carrying *C. brevicomi*. We present evidence that throughout the flight season, over broad geographic distances, and among genetically distinct populations of beetle, the western pine beetle is associated with the same two species of fungi. In addition, we provide evidence that one fungal species is associated with larger adult beetles and therefore might provide greater benefit during beetle development. The importance and maintenance of this bark beetle-fungus interaction is discussed.

INTRODUCTION

Symbioses have supported the evolution of much of the biological diversity on earth [1]. Insect-microbe symbioses have not only supported insect diversification, but have also facilitated their ecological dominance in some communities, allowing some to rank as ecosystem engineers. Some of the most ecologically and economically important insects are involved in mutualistic relationships with either bacteria [2] or fungi [3-5].

A long standing question in the study of insect-microbe mutualisms is whether these relationships are maintained for long periods of time or are more labile with flexibility in partner acquisition [6, 7]. Much of symbiotic theory suggests that mutualisms are inherently unstable and prone to cheating which should erode the interaction over time [6-8]. However, many mutualisms have remained stable over long time periods. Strict vertical transovarial transmission of the symbiont from parent to offspring often enables stability over evolutionary time frames [9, 10]. Vertical transmission is common in insect-bacterial endosymbioses which tend to be stable over tens to hundreds of millions of years [11, 12]. However, with ectosymbiotic “fungus-farming” insects, the symbionts often live independent of the host for a period of time and hosts are sometimes exposed to ‘pools’ of potential alternate symbionts during their development [4, 5, 13]. While many fungus-farming insects have evolved mechanisms that increase the probability of vertical transmission, horizontal transmission does occur and may have repercussions on the long term stability and potential for coevolution and cospeciation among partners [14]. Several studies suggest that even the most derived forms of insect-fungal farming may have experienced some degree of symbiont replacement over evolutionary time [15-18] although some systems appear to have remained quite stable and show evidence of coevolution and cospeciation [19].

One group of insects well known for their associations with fungi are bark beetles (Curculionidae, Scolytinae) [5, 20-22]. Considered to be among of the most ecologically important organisms in forest ecosystems worldwide, all are associated with fungi to some extent. A small number of beetle species are known to be involved in highly specialized mutualisms and have evolved exoskeletal structures (termed mycangia) for transporting their fungal partners [23, 24]. In these systems, the insect benefits primarily from gaining nutrients from fungal feeding while the fungus benefits by gaining transportation to new resources [25, 26]. These beetle-fungal interactions involve vertical transfer of the symbiont from parent to offspring via the mycangia, thereby providing continuity of the interaction [5]. However, it is not known how 'leaky' these transfers may be and how prone they are to invasion [5]. Even rare invasion events may result in additions or substitutions that may alter symbiont communities at local or broad scales.

Most bark beetle-fungal symbioses are not well characterized, very few have been surveyed over the range of the host, and of those, only beetle species with maxillary mycangia have been investigated [27-29]. It is also not known if and how these relationships are maintained at ecological and evolutionary time scales [28-30]. One system well suited for addressing these questions is the western pine beetle (*Dendroctonus brevicomis* LeConte) (hereafter WPB). WPB is one of the most important forest insects in the western United States causing annual tree losses ranging from 500 to 3,500 million board feet of timber [31]. WPB specializes on ponderosa pine (*Pinus ponderosa* Douglas) and Coulter pine (*Pinus coulteri* D. Don) with the beetle's distribution generally corresponding to that of its hosts [32]. The beetle kills the tree through a pheromone-mediated mass attack and the eggs are laid in the phloem layer where the larvae spend the first and second instars feeding. Subsequent instars move into

the outer bark to complete development [31]. The nutrient poor feeding substrate of late instar larvae combined with the presence of mycangia and fungal symbionts strongly suggests this beetle depends on its fungi for nutritional supplementation.

Female beetles possess a prothoracic mycangium that is used to transport the fungal symbionts [33]. The mycangium consists of two partitioned compartments which can independently carry one, both, or neither fungal species. The mycangial fungi have been described as *Entomocorticium* sp. B, a basidiomycete, and *Ceratocystiopsis brevicomi*, an ascomycete [33-36]. Both fungi are highly specific to the beetle and are morphologically adapted for insect dispersal [35, 36]. While the effects of the fungi on the host beetle have not been investigated, the fungi are predicted to fulfill roles similar to the fungal mutualists of *D. frontalis*, a closely related species that carries closely related fungi, *E.* sp. A and *C. ranaculosus*, and possesses a similar prothoracic mycangium [37, 38]. Field and laboratory-based studies have shown that *E.* sp. A is a superior symbiotic partner to *C. ranaculosus*, as adult *D. frontalis* developing with this fungus are larger and possess greater amounts of lipids [39-41]. Temporal fluctuations occur in the relative abundance of the fungal mutualists and *Entomocorticium* sp. A is isolated from beetles more frequently during warmer parts of the season while *C. ranaculosus* is more prevalent during cooler periods [42]. It is not known if the fungi associated with WPB are similarly affected by temperature and fluctuate in prevalence seasonally.

Reported isolations of fungi from WPB are rare and it is unclear whether *E.* sp. B and *C. brevicomi* are associated with the beetle across its extensive geographic range. Furthermore, population genetic analyses suggest the WPB is actually two cryptic species [43]. Beetles from the Southwest (UT, AZ, NM, CO) are thought to have last shared a common ancestor with beetles in the westernmost part of the range (CA, OR, ID, and BC Canada) several million years

ago [43]. Cryptic beetle species are isolated on two ponderosa pine subspecies thought to have formed in glacial refugia during the Pleistocene [44]. The tree subspecies are not only geographically disjunct, but also differ in host tree defensive chemistry [45], which may exert strong selection on both beetles [46] and fungi [47]. Differences in host beetles and trees may also be reflected in divergence of their fungal symbionts or could result in symbiont swapping.

Our overall objective was to evaluate fungal fidelity in the WPB-fungus symbiosis. Specifically, we wished to determine 1) the identity and distribution of fungi associated with the mycangia of WPB across its range, 2) the genetic structure of the mycangial fungi, and whether it corresponds to cryptic beetle species, 3) whether the fungi fluctuate in prevalence over a flight season, and 4) if the symbionts may differentially benefit the host.

METHODS

Beetle-fungus collection: WPB populations were sampled across the beetle's geographic range in the summer of 2011. Beetles were collected in one of three ways: 1) trapped via Lindgren funnel traps baited with WPB attractant lures (Synergy Semiochemicals Corp; part# P130 WPB trap lure, or special "Southwest WPB" lure [48]), 2) rearing from bark from beetle-infested ponderosa pine trees (primarily 4th instar larvae, pupae and teneral adults), or 3) by physically removing adult beetles from trees during the early stages of colonization. When bark was collected, multiple infested trees were sampled at various positions on the lower bole in an attempt to sample brood from different parents. Bark samples were held in rearing containers at ~21° C until adult emergence. Field collected adult beetles were transported live and kept at ~4 °C prior to dissection and isolation of mycangial fungi.

Fungal isolation: We used culture-based methods for fungal isolation and screening. These methods are routinely used to identify fungi associated with bark beetles [27, 49-51] and are particularly appropriate for some mycangium-bearing species, and especially WPB, as only a few spores may be present in the structure [33] which may yield too little DNA for use with standard sequence-based species identification.

Live female WPB were distinguished from males using morphological features of the head and the 7th abdominal tergite [52] and presence of the prothoracic mycangial swelling [32]. Beetles were then surface sterilized to remove phoretic fungi and dissected similarly to Davis et al. [53] with a few modifications. Whole beetles were first immersed in 70% ethanol and shaken by hand vigorously for 45 s, then transferred to sterilized deionized water, shaken vigorously for 45 s, and then allowed to dry in a sterile Petri dish. The mycangium was then dissected from the thorax by removing the head, cutting the thorax dorsoventrally, and removing the anterior portion of the two pronotal sections containing each mycangium. Both sections of the mycangium from each beetle was placed on opposite sides of a 90 mm Petri dish containing 2% malt extract agar (MEA) and held at room temperature (~21 °C) for about 6 wk to facilitate fungal growth.

Fungal identification: Subcultures of mycelial growth were taken from the cultures initiated from mycangia. The resulting subcultures were placed into morphogroups based on color and growth characteristics. Two filamentous fungi that have previously been isolated from the mycangia of WPB are visually distinct. One is a basidiomycete and produces “brown to light gray-brown” mycelia when grown on MEA [36] while the other is an ascomycete and is “cream” colored with a morphology distinct from the basidiomycete [35]. For each collection location,

subcultured fungi were first grouped into morphotypes. Then a representative culture from each morphotype was subcultured again to purify it for use in DNA extraction and sequencing. In addition, reference cultures from Hsiau and Harrington [36] of *E.* sp. B (isolate B1037), and its close relatives *E*. sp. C (isolate B896), *E*. sp. H (isolate B1065) and *E*, sp. G (isolate B1069), were obtained from Tom Harrington, Iowa State University, for morphological and DNA sequence-based comparisons. Reference isolates of *E.* sp. A and *C. ranaculosus* were obtained from *D. frontalis* collected in Florida (30° 11' N, 81° 23' W) and Arizona (33° 28' N, 109° 22' W).

DNA extraction was performed by scraping mycelium from the surface of the cultures, grinding the tissue in a microcentrifuge tube with sterile sand to rupture hyphal walls, followed by the standard extraction methods provided in the PrepMan Ultra protocol (Applied Biosystems, Foster City, CA, USA). Prior to screening samples for species identification, reference sequences were obtained for *Entomocorticium* spp. and *Ceratocystiopsis* spp. in GenBank (Accessions AF1190503 – AF1190512, EU913721 EU913722, and EU913713). The ITS2-LSU region was determined to be diagnostic for *Entomocorticium* as taxa differed at multiple nucleotide positions. However, the closely related *C. brevicomi* and *C. ranaculosus* were more difficult to differentiate, so the more variable beta-tubulin region was sequenced for these fungi. For the *Entomocorticium* isolated from WPB in this study, the primer pair ITS3 [54] - LR3 [55] was used to amplify the ITS2 and LSU region of rDNA. For the *Ceratocystiopsis* that was isolated, the Bt2b [56] - T10 [57] primer pair was used. PCR conditions for the ITS2-LSU sequencing were 92 °C for 2 min (95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min) X40 with a final 8 min 72 °C extension while conditions for beta-tubulin were 95 °C for 1 min (95 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min) X35 and a final 72 °C extension for 5 min. PCR products

were cleaned using a High Pure PCR product purification kit (Roche Molecular Biochemicals, Indianapolis, USA) and sequenced on an ABI 3130 automated sequencer at the Murdock Sequencing Facility (University of Montana, Missoula, MT, USA).

Sequences were inspected by eye, edited using MEGA5 [58], and searched against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST. For phylogenetic comparisons between sequenced isolates and their best matches in GenBank (*Ceratocystiopsis* comparisons) or reference isolates (*Entomocorticium* comparisons), all sequences were first aligned using MAFFT v.7 [59] using default parameters. Phylogenetic analyses were then conducted using maximum likelihood, with 500 bootstrap replicates and GTR + Gamma model of nucleotide substitution using RAxML v.7.2.8 [60]. All unique sequences have been deposited in GenBank (Accessions KJ620518 – KJ620531) and a representative isolate deposited in the CBS Fungal Diversity Center database (Accessions CBS137840 – CBS137853).

Temporal prevalence of WPB mycangial fungi during dispersal: To investigate whether the mycangial fungi of WPB fluctuate in prevalence during the flight season, and to determine whether a single collection event would provide a representative sample of the fungi associated with the beetle at a site, beetles were collected over the flight season from two sites in 2012 that had been sampled in 2011. At the Missoula and Greenough sites (Table 1), Lindgren funnel traps baited with WPB specific lures (Synergy Semiochemical Corp; part# P130 WPB trap lure) were deployed on 7 May 2012, for the Missoula site, and 30 May 2012, for the Greenough site. Traps were established before WPB initiated flights at these sites (based on inspection of brood in trees attacked the previous summer/fall). To characterize the thermal environment at out sites, and to determine if temperature may play role the abundance of a particular fungal partner, daily

minimum and maximum temperatures were estimated for each site by interpolating from nearby weather stations [Oyler et al. in revision]. Up to 50 female beetles per collection day, per site, were surface sterilized, dissected and the mycangium plated on 2% MEA as previously described. Each beetle was scored for presence or absence of *E.* sp. B and *C. brevicomi* in the mycangia.

Relationship between fungal species and beetle size: To investigate whether the two fungi might differentially affect female beetle size (a component of fitness), prior to dissection and plating of mycangia, female beetles were assessed for size. The pronotum width (a proxy measure of body size) was determined for all females by capturing digital images using a Leica EZ4 D stereo microscope with built in 3-megapixel camera. Digital images were then analyzed in ImageJ [61]. To explore the relationship between fungal species and body size, linear models were fit to the data using R v. 3.0.1 (R Development Core Team 2011). Body size measurements were checked for normality and were found to be left skewed. The optimal transformation based on the Box-Cox procedure [62] in the R *MASS* package was to raise body size to 4th power, which normalized the data and met linear model assumptions. To account for any influence on size that collection date might have, the collection date was included as a covariate as was the interaction between fungal species and collection date. Post-hoc comparisons between fungal species and body size were conducted in the R *multcomp* package using Tukey's HSD (Honestly Significant Difference) tests.

RESULTS

A total of 591 beetles were collected from 29 geographically distinct locations (Table 1). Twenty-seven beetles (4.6%) yielded no fungi from their mycangium. Ninety-six beetles (16.2%) had significant contamination with environmental fungi or yeasts, which prevented reliable subculturing of mycangial fungi. This left a total of 468 beetles (79.2%) ($\bar{x}$ ~ 16 per sampling location) that yielded cultures of mycangial fungi. Of these, 66.9% of beetles were found to exclusively carry fungi morphotyped as *Entomocorticium*, while 25.9% exclusively carried the *Ceratocystiopsis* morphotype, and 7.3% were found to carry both (Fig. 1). Within a sampling location, the proportion of beetles that exclusively carried one or the other morphotype varied considerably. Notably, multiple collection locations in the Southwest included little to no *Ceratocystiopsis*, while this morphotype was isolated with equal frequency as *Entomocorticium* in some northwest populations (Fig. 1).

A 543 bp region of the Beta-tubulin gene was sequenced for 23 *Ceratocystiopsis* isolates, each from a different collection location. The top BLAST search matches for these sequences exhibited 99% -100% sequence identity (query coverage 94%) with *C. brevicomi* (Accession EU913761). Phylogenetic comparisons conducted with all *Ceratocystiopsis* sequences available in GenBank where query coverage was > 80%, and sequence identity was > 80% placed all isolates sequenced in this study clearly within *C. brevicomi* (Fig. 2). Furthermore, the inferred relationships among these species strongly suggest that the mycangial fungus of the southern pine beetle, *C. ranaculosus* is the closest known relative to *C. brevicomi* (Fig. 2). There was a high degree of sequence similarity among the *C. brevicomi* isolates; five haplotypes were identified (Table 2), which in total possessed only five variable sites and no indels. No clear

phylogeographic or population genetic structuring among the C. *brevicomi* haplotypes was observed (Table 2, Supplementary file 1).

A 765 bp region of the ITS2-LSU region was sequenced for 29 *Entomocorticium* isolates, (one from each collection area). The top BLAST search match, based on percent sequence identity, was an *Entomocorticium* sp. identified by Davis et al. [53] from WPB (Accession HQ413289, strain AZF001). All 29 sequenced isolates shared 99% identity (61% query coverage) with this strain. Genetic variation within the isolates was small. Only three haplotypes were identified (Table 2), with only three variable positions, and no indels. There was some evidence of phylogeographic structuring as haplotype C was only found in samples collected from the Southwest (Fig. 3, Supplementary file 1). The other two haplotypes, A and B, showed no clear geographic pattern in western populations (Fig. 3, Supplementary file 1). To further explore if certain sample locations are dominated by one particular haplotype, four collection areas, Missoula, La Grande, Darby, and Prineville (Table 1), had an additional 2-4 isolates sequenced per location. A mixture of both B and C haplotypes were found within each of these collection areas (Table 2, Supplementary file 1). Comparative analyses of sequences from type cultures of *Entomocorticium* species A, B, C, G, and H, and the 29 isolates from different collection areas found that haplotype A was a perfect match to reference *E.* sp. B, isolate B1037 [36]. Phylogenetic analyses clustered all isolates from WPB into a well-supported monophyletic group which included the *E.* sp. B reference isolate B1037 (Fig. 3).

Fungal prevalence over time: At the Greenough site, 198 beetles were sampled at five dates from 12 June to 28 August 2012 (average number of beetles per sampling date, ~39). In total, 148 (75.0%) of these beetles carried only *E.* sp. B, 11 (5.5%) carried only *C. brevicomi*, 15 (7.5%) carried both fungi, 3 (1.5%) had no fungi, and 21 (10.5%) were contaminated. At the

Missoula site, 313 beetles were sampled eight times from 12 May to 12 October 2012 (average per sampling date, ~39). In total, 188 (60.0%) exclusively carried *E.* sp. B, 42 (13.5%) exclusively carried *C. brevicomi*, 26 (8.5%) carried both fungi, 4 (1.0%) had no fungi, and 53 (17.0%) were contaminated. On any given sampling date at either collection site, ≥ 60% of WPB were found to be exclusively carrying *E.* sp. B, while the percentage that only carried *C. brevicomi* was never greater than 30% (Fig. 4). No clear temporal trend of increasing or decreasing abundance of either fungal species over the process of the flight season was observed at either site (Fig. 4). In particular, cooler periods, most apparent at the Missoula site in the spring and fall, did not correspond to a clear increase in the abundance of *C. brevicomi* (Fig. 4).

Beetle size related to fungal species: The relationship between mycangial fungi and body size was analyzed by grouping the beetles into three fungal classes: beetles where 1) only *E.* sp. B was isolated, 2) only *C. brevicomi* was isolated, and 3) where both species were isolated. At the Missoula site, there was a significant effect of fungal class ($F_{2,234} = 7.35$, $p < 0.0005$) on body size in the linear model. Collection date was also statistically significant and greatly improved model fit ($F_{1,234} = 22.48$, $p < 0.0005$) as beetles collected later in the season were on average smaller. There was no significant interaction between fungal class and body size ($F_{2,234} = 1.17$, $p = 0.3105$). After accounting for the effect of the collection date, Tukey HSD pairwise comparisons between beetles of different fungal classes identified that beetles carrying *E.* sp. B were significantly larger than beetles carrying either *C. brevicomi*, or both species (Fig. 5a). At the Greenough site, there was also a significant effect of fungal class on body size ($F_{2,170} = 5.15$, $p = 0.0067$), although collection date did not have a statistically significant effect ($F_{1,170} = 2.55$, $p = 0.1121$). The interaction between these two variables was not analyzed as *C. brevicomi* was isolated from too few sampling dates to properly test for this effect. Post-hoc Tukey HSD

pairwise comparisons indicated that beetles carrying *E.* sp. B were again significantly larger than beetles carrying *C. brevicomi*, while beetles carrying both species did not significantly differ in size from beetles exclusively carrying one or the other fungus (Fig. 5b).

DISCUSSION

The results of this study conclusively demonstrate that the mycangial fungi of the WPB are *E.* sp. B and *C. brevicomi*. Our findings indicate that this multipartite symbiosis is maintained across the large geographic distribution of the WPB and among highly genetically differentiated beetle populations [43]. Additionally, *E.*sp B and *C. brevicomi* are found in beetles that colonize ponderosa pine subspecies with substantial differences in defensive chemistry [45]. It is also notable given that WPB often co-colonize host trees with other bark beetles and, thus, are exposed to a substantial alternate symbiont pool [63]. For example, in the Southwestern US, ponderosa pine is often co-colonized by *D. approximatus*, *D. adjunctus*, and *D. frontalis,* all of which have prothoracic mycangia, and closely related fungal symbionts. In other portions of the WPB range, trees are often co-colonized by *D. ponderosae*, another species closely associated with fungi but with a different type of mycangium [64]. Our observation that the WPB exhibits a high level of fungal partner fidelity is consistent with findings from the two other *Dendroctonus* species where geographically broad scale surveys have been conducted [27-29]. However, our study is the first to demonstrate this fidelity in a species with a prothoracic myangium. Conservatism and continuity in the insect-fungal interaction is imperative for long-term coevolution between insect and their mutualist symbiont [14]. Our results suggest a tightly linked obligate relationship between the WPB and its mycangial fungi with the potential for a long-term association. Currently, it is unknown how such strict fidelity is maintained, particularly in light

of continual exposure to other beetle-associated fungi. One tantalizing hypothesis is that the fine structures and glandular secretions of the prothoracic mycangia may play a role in filtering and selecting suitable symbionts [23, 24].

Most bark beetle-fungal symbioses are multipartite, with each fungal species conferring different degrees of benefit to the beetle [26]. Consistent with studies of *D. frontalis* mycangial fungi that have found that *E.* sp. A provides a greater degree of benefit to the beetle than does *C. ranaculosus* [39-41], *E.* sp. B appears to provide greater benefit to WPB than does *C. brevicomi*. Females that were captured in traps carrying only *E.* sp. B were significantly larger at both sites in our study. Given the general positive relationship in insects between body size and fecundity [65], females that develop in an environment dominated by *E.* sp. B are more likely to have more offspring and increased fitness. In our study, *E.* sp. B was found to be more prevalent than *C. brevicomi* throughout the season at both collection sites. These findings are in contrast to what is observed in *D. frontalis* where there are clear seasonal fluctuations in the relative abundance of the two symbionts, and *C. ranaculosus* is more prevalent in the cooler parts of the season, while *E.* sp. A is more prevalent in warmer parts [42]. Likewise, the two mycangial fungi associated with *D. ponderosae* fluctuate as temperatures shift over time [66]. In our collections across the range of the WPB, we identified some locations where *C. brevicomi* appears to be more prevalent than *E.* sp. B, however, it is unclear if this was an artifact of sampling at only one time point and/or small sample sizes. Further sampling is needed to understand how symbionts may fluctuate within populations over time and to understand how local environmental conditions may favor particular fungal symbionts, which could, in turn, affect beetle population dynamics given the differing level of benefit that each symbiont provides.

Given that *E.* sp. B appears to be the better symbiotic partner, was the most frequently encountered mycangial fungus across the range of WPB, and dominated collections throughout the season at our two sites in Montana, the question arises as to how *C. brevicomi* is maintained in the symbiosis. Indeed, there were multiple areas in the Southwest where we did not detect *C. brevicomi* in our collections, and others have observed that this fungus is an infrequent associate of the beetle in this region (Seth Davis, pers. comm.). One possible explanation is that although *E.* sp. B is the better symbiotic partner, *C. brevicomi* may have slightly different environmental tolerances, and therefore, a different fundamental niche. This may allow WPB to maintain symbiotic relationships with nutrient-providing fungi over a much larger range of environmental conditions, even if the benefits from *C. brevicomi* are less pronounced. There is evidence of such a scenario in *D. ponderosae* where two symbiotic fungi provide different levels of benefit to the beetle [26], and grow maximally at different temperatures [67]. In the field, it has been shown that at warmer sites, the warm-tolerant fungus predominates, while at cooler sites, the other fungus predominates [66]. Simulations suggest that seasonal temperature fluctuations plus occasional migration between different thermal habitats can act to maintain both symbionts in the mutualism [68]. In the WPB, environmental heterogeneity may help maintain these associations at both ecological and evolutionary time scales, but the role that temperature alone might play remains unclear. Biotic interactions are also important and can influence the relative abundance of either fungal species. Host tree defenses vary dramatically across the range of ponderosa pine [45] and which may differentially impact *E.* sp. B and *C. brevicomi*. Mites and nematodes that co-occur with bark beetles may also influence the abundance of particular fungal species [42].

The levels of 'contaminant' fungi in our study were not surprising given that our methods were not intended to be selective or our surface sterilization techniques overly harsh. Many culture-based methods that survey the fungal associates of bark beetles often use cycloheximide-amended media to select for Ophiostomatales and/or lengthy alcohol rinses to reduce the quantity of contaminating microbes. We purposely used a short alcohol rinse as it has been shown that lengthy washes decreased the probability of isolating *Ceratocystiopsis* [35]. We also did not amend our growth medium with cyclohexamide as it is known to inhibit the growth of basidiomycetes including *Entomocorticium*. Culture-based methods are known to be biased, and the possibility exists that some fungi that occur infrequently in the mycangia either failed to grow, or were misdiagnosed as contaminant fungi [50]. However, we feel these biases were minimal in our study for several reasons. During culturing, every attempt was made to subculture morphologically distinct fungi from different time points during the growth phase so as to capture slow growing species that would otherwise be overgrown by faster growing fungi. Additionally, the two mycangial fungi of the WPB have very similar, and slow growth rates at room temperature [37], and there were multiple cases where both species of fungi were recovered from one mycangia. Finally, culture independent methods used to estimate the frequency of *E.* sp. A and *C. ranaculosus* in *D. frontalis* have resulted in very similar estimates [39, 42].

The identification of three distinct haplotypes of *E.* sp. B together with some geographic structuring consistent with WPB cryptic species is suggestive of some co-divergence of beetle and fungus. One haplotype was collected only from beetles from the Southwest, while the other two appear to be intermixed in the westernmost populations. There are limitations with single gene phylogenetic analyses of mycangial fungi [28, 69] and conclusions about the placement of

E. sp. B in the larger, and poorly described, *Entomocorticium* group need to be taken with caution. To clarify evolutionary relationships and identify whether any cryptic species of either *Ceratocystiopsis* or *Entomocorticium* are present will require a more comprehensive genetic analysis using additional regions of the genome. The slight genetic variation observed between different *Entomocorticium* haplotypes could be due to intragenomic variation that is known to occur in rDNA [70]. Population genomic analyses of these *Entomocorticium* and *Ceratocystiopsis* isolates are currently underway and will begin to establish phylogenetic patterns among these fungi.

In conclusion, our finding of such strict insect-fungal fidelity speaks to the strong mutualistic ties between WPB, *C. brevicomi* and *E.* sp. B. This level of fidelity is a crucial ingredient for any coevolution and cospeciation to occur between these organisms [14, 19]. Future research aimed at describing the fine scale population genetic structure and evolutionary history of both fungi and beetle will provide much needed information about symbiont movement, life history, and patterns of codifferentiation between beetle and fungus.

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REFERENCES CITED

1. Margulis, L. and R. Fester, *Symbiosis as a source of evolutionary innovation*. 1991, Cambridge, MA: MIT Press.
2. Douglas, A.E., *Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria Buchnera.* Annual Review of Entomology, 1998. **43**: p. 17-37.
3. Wilding, N., et al., *Insect-Fungus Interactions*. 1989, London: Academic Press.
4. Mueller, U.G., et al., *The evolution of agriculture in insects.* Annual Review of Ecology Evolution and Systematics, 2005. **36**: p. 563-595.
5. Six, D.L., *Ecological and evolutionary determinants of bark beetle—fungus symbioses.* Insects, 2012. **3**(1): p. 339-366.
6. Herre, E.A., et al., *The evolution of mutualisms: exploring the paths between conflict and cooperation.* Trends in Ecology & Evolution, 1999. **14**(2): p. 49-53.
7. Sachs, J.L., et al., *The evolution of cooperation.* Quarterly Review of Biology, 2004. **79**(2): p. 135-160.
8. Ferriere, R., et al., *Cheating and the evolutionary stability of mutualisms.* Proceedings of the Royal Society B-Biological Sciences, 2002. **269**(1493): p. 773-780.
9. Ewald, P.W., *Transmission Modes and Evolution of the Parasitism-Mutualism Continuum.* Annals of the New York Academy of Sciences, 1987. **503**: p. 295-306.

10. Moran, N.A., *Symbiosis as an adaptive process and source of phenotypic complexity.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**: p. 8627-8633.

11. Clark, M.A., et al., *Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence.* Evolution, 2000. **54**(2): p. 517-525.

12. Conord, C., et al., *Long-term evolutionary stability of bacterial endosymbiosis in curculionoidea: Additional evidence of symbiont replacement in the dryophthoridae family.* Molecular Biology and Evolution, 2008. **25**(5): p. 859-868.

13. Aanen, D.K., et al., *High symbiont relatedness stabilizes mutualistic cooperation in fungus-growing termites.* Science, 2009. **326**(5956): p. 1103-6.

14. Janson, E.M., et al., *Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification.* Evolution, 2008. **62**(5): p. 997-1012.

15. Aanen, D.K., et al., *The evolution of fungus-growing termites and their mutualistic fungal symbionts.* Proc Natl Acad Sci USA, 2002. **99**(23): p. 14887-92.

16. Mikheyev, A.S., U.G. Mueller, and P. Abbot, *Cryptic sex and many-to-one colevolution in the fungus-growing ant symbiosis.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(28): p. 10702-10706.

17. Mikheyev, A.S., U.G. Mueller, and J.J. Boomsma, *Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi.* Molecular Ecology, 2007. **16**(1): p. 209-216.

18. Mikheyev, A.S., T. Vo, and U.G. Mueller, *Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists.* Molecular Ecology, 2008. **17**(20): p. 4480-4488.

19. Mehdiabadi, N.J., et al., *Symbiont fidelity and the origin of species in fungus-growing ants.* Nature Communications, 2012. **3**.

20. Paine, T.D., K.F. Raffa, and T.C. Harrington, *Interactions among scolytid bark beetles, their associated fungi, and live host conifers.* Annual Review of Entomology, 1997. **42**: p. 179-206.

21. Six, D.L. and K.D. Klepzig, *Dendroctonus bark beetles as model systems for studies on symbiosis.* Symbiosis, 2004. **37**(1-3): p. 207-232.

22. Harrington, T.C., *Ecology and evolution of mycophagous bark beetles and their fungal partners*, in *Ecological and Evolutionary Advances in Insect-Fungal Associations*, F.E. Vega and M. Blackwell, Editors. 2005, Oxford University Press. p. 257-291.

23. Batra, L.R., *Ecology of ambrosia fungi and their dissemination by beetles.* Transactions of the Kansas Academy of Science (1903), 1963: p. 213-236.

24. Yuceer, C., et al., *Ultrastructure of the mycangium of the southern pine beetle, Dendroctonus frontalis (Coleoptera: Curculionidae, Scolytinae): complex morphology for complex interactions.* Acta Zoologica, 2011. **92**(3): p. 216-224.

25. Ayres, M.P., et al., *Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi.* Ecology, 2000. **81**(8): p. 2198-2210.

26. Bleiker, K.P. and D.L. Six, *Dietary benefits of fungal associates to an eruptive herbivore: Potential implications of multiple associates on host population dynamics.* Environmental Entomology, 2007. **36**(6): p. 1384-1396.

27. Six, D.L. and T.D. Paine, *Ophiostoma clavigerum is the mycangial fungus of the Jeffrey pine beetle, Dendroctonus jeffreyi.* Mycologia, 1997. **89**(6): p. 858-866.
28. Alamouti, S.M., et al., *Gene genealogies reveal cryptic species and host preferences for the pine fungal pathogen Grosmannia clavigera.* Mol Ecol, 2011. **20**(12): p. 2581-602.
29. Roe, A.D., et al., *Comparative phylogeography, genetic differentiation and contrasting reproductive modes in three fungal symbionts of a multipartite bark beetle symbiosis.* Molecular Ecology, 2011. **20**(3): p. 584-600.
30. James, P.M.A., et al., *Spatial Genetic Structure of a Symbiotic Beetle-Fungal System: Toward Multi-Taxa Integrated Landscape Genetics.* Plos One, 2011. **6**(10).
31. Miller, J.M. and F.P. Keen, *Biology and control of the western pine beetle: a summary of the first 50 years of research*. 1960, Washington, DC: U.S. Department of Agriculture.
32. Wood, S.L., *The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae).* Great Basin Naturalist, 1982. **6**: p. 1-1359.
33. Paine, T.D. and M.C. Birch, *Acquisition and Maintenance of Mycangial Fungi by Dendroctonus-Brevicomis Leconte (Coleoptera, Scolytidae).* Environmental Entomology, 1983. **12**(5): p. 1384-1386.
34. Whitney, H.S. and F.W. Cobb, *Non-Staining Fungi Associated with Bark Beetle Dendroctonus Brevicomis (Coleoptera-Scolytidae) on Pinus Ponderosa.* Canadian Journal of Botany, 1972. **50**(9): p. 1943-&.
35. Hsiau, P.T.W. and T.C. Harrington, *Ceratocystiopsis brevicomi sp nov, a mycangial fungus from Dendroctonus brevicomis (Coleoptera: Scolytidae).* Mycologia, 1997. **89**(4): p. 661-669.

36. Hsiau, P.T.W. and T.C. Harrington, *Phylogenetics and adaptations of basidiomycetous fungi fed upon by bark beetles (Coleoptera : Scolytidae).* Symbiosis, 2003. **34**(2): p. 111-131.

37. Davis, T.S., et al., *Interactions between multiple fungi isolated from two bark beetles, Dendroctonus brevicomis and Dendroctonus frontalis (Coleoptera: Curculionidae).* Journal of Yeast and Fungal Research, 2010. **1**: p. 118-126.

38. Klepzig, K.D. and R.W. Hofstetter, *From attack to emergence: interactions between the southern pine beetle, mites, microbes, and trees*, in *Southern pine beetle II*, R.N. Coulson and K.D. Klepzig, Editors. 2011, USDA Forest Service Gen. Tech. Rep. SRS-140. p. 141-152.

39. Bridges, J.R., *Mycangial Fungi of Dendroctonus-Frontalis (Coleoptera, Scolytidae) and Their Relationship to Beetle Population Trends.* Environmental Entomology, 1983. **12**(3): p. 858-861.

40. Goldhammer, D.S., F.M. Stephen, and T.D. Paine, *The Effect of the Fungi Ceratocystis-Minor (Hedgecock) Hunt, Ceratocystis-Minor (Hedgecock) Hunt Var Barrasii-Taylor, and Sjb-122 on Reproduction of the Southern Pine-Beetle, Dendroctonus-Frontalis Zimmermann (Coleoptera, Scolytidae).* Canadian Entomologist, 1990. **122**(5-6): p. 407-418.

41. Coppedge, B.R., F.M. Stephen, and G.W. Felton, *Variation in Female Southern Pine-Beetle Size and Lipid-Content in Relation to Fungal Associates.* Canadian Entomologist, 1995. **127**(2): p. 145-154.

42. Hofstetter, R.W., et al., *Seasonal dynamics of mites and fungi and their interaction with Southern Pine Beetle.* Environmental Entomology, 2006. **35**(1): p. 22-30.

43. Kelley, S.T., J.B. Mitton, and T.D. Paine, *Strong differentiation in mitochondrial DNA of Dendroctonus brevicomis (Coleoptera : Scolytidae) on different subspecies of ponderosa pine.* Annals of the Entomological Society of America, 1999. **92**(2): p. 193-197.
44. Latta, R.G. and J.B. Mitton, *Historical separation and present gene flow through a zone of secondary contact in ponderosa pine.* Evolution, 1999. **53**(3): p. 769-776.
45. Smith, R.H., *Monoterpenes of ponderosa pine xylem resin in western United States*. 1977, USDA. p. 1-48.
46. Sturgeon, K.B., *Monoterpene Variation in Ponderosa Pine Xylem Resin Related to Western Pine-Beetle Predation.* Evolution, 1979. **33**(3): p. 803-814.
47. Davis, T.S. and R.W. Hofstetter, *Plant secondary chemistry mediates the performance of a nutritional symbiont associated with a tree-killing herbivore.* Ecology, 2012. **93**(2): p. 421-429.
48. Hofstetter, R.W., et al., *Synergistic effects of alpha-pinene and exo-brevicomin on pine bark beetles and associated insects in Arizona.* Journal of Applied Entomology, 2008. **132**(5): p. 387-397.
49. Lee, S., J.J. Kim, and C. Breuil, *Leptographium longiclavatum sp nov., a new species associated with the mountain pine beetle, Dendroctonus ponderosae.* Mycological Research, 2005. **109**: p. 1162-1170.
50. Roe, A.D., et al., *Spatial Community Structure of Mountain Pine Beetle Fungal Symbionts Across a Latitudinal Gradient.* Microbial Ecology, 2011. **62**(2): p. 347-360.
51. Biedermann, P.H.W., et al., *Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle Xyleborinus saxesenii Ratzeburg*

(Coleoptera: Curculionidae, Scolytinae). Fems Microbiology Ecology, 2013. **83**(3): p. 711-723.

52. Lyon, R., *A useful secondary sex character in Dendroctonus bark beetles.* The Canadian Entomologist, 1958. **90**(10): p. 582-584.

53. Davis, T.S., et al., *Interactions Between the Yeast Ogataea pini and Filamentous Fungi Associated with the Western Pine Beetle.* Microbial Ecology, 2011. **61**(3): p. 626-634.

54. White, T.J., et al., *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*, in *PCR Protocols: A Guide to Methods and Applications*, M.D. Innis, et al., Editors. 1990, Academic Press, Inc.: New York. p. 315-322.

55. Vilgalys, R. and M. Hester, *Rapid Genetic Identification and Mapping of Enzymatically Amplified Ribosomal DNA from Several Cryptococcus Species.* Journal of Bacteriology, 1990. **172**(8): p. 4238-4246.

56. Glass, N.L. and G.C. Donaldson, *Development of Primer Sets Designed for Use with the Pcr to Amplify Conserved Genes from Filamentous Ascomycetes.* Applied and Environmental Microbiology, 1995. **61**(4): p. 1323-1330.

57. O'Donnell, K. and E. Cigelnik, *Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus Fusarium are nonorthologous.* Mol Phylogenet Evol, 1997. **7**(1): p. 103-16.

58. Tamura, K., et al., *MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods.* Molecular Biology and Evolution, 2011. **28**(10): p. 2731-2739.

59. Katoh, K. and D.M. Standley, *MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability.* Molecular Biology and Evolution, 2013. **30**(4): p. 772-780.

60. Stamatakis, A., *RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models.* Bioinformatics, 2006. **22**(21): p. 2688-2690.

61. Abràmoff, M.D., P.J. Magalhães, and S.J. Ram, *Image processing with ImageJ.* Biophotonics international, 2004. **11**(7): p. 36-43.

62. Box, G.E.P. and D.R. Cox, *An Analysis of Transformations.* Journal of the Royal Statistical Society Series B-Statistical Methodology, 1964. **26**(2): p. 211-252.

63. Davis, T.S. and R.W. Hofstetter, *Effects of Gallery Density and Species Ratio on the Fitness and Fecundity of Two Sympatric Bark Beetles (Coleoptera: Curculionidae).* Environmental Entomology, 2009. **38**(3): p. 639-650.

64. Davis, R.S., S. Hood, and B.J. Bentz, *Fire-injured ponderosa pine provide a pulsed resource for bark beetles.* Canadian Journal of Forest Research-Revue Canadienne De Recherche Forestiere, 2012. **42**(12): p. 2022-2036.

65. Honek, A., *Intraspecific Variation in Body Size and Fecundity in Insects - a General Relationship.* Oikos, 1993. **66**(3): p. 483-492.

66. Six, D.L. and B.J. Bentz, *Temperature determines symbiont abundance in a multipartite bark beetle-fungus ectosymbiosis.* Microbial Ecology, 2007. **54**(1): p. 112-118.

67. Moore, M.L., *The effects of temperature on fungal symbionts in the mountain pine beetle-fungus multi-partite symbiosis*, in *Ecosystem and Conservation Sciences*. 2013, University of Montana: University of Montana.

68. Addison, A.L., et al., *The role of temperature variability in stabilizing the mountain pine beetle-fungus mutualism.* J Theor Biol, 2013. **335**: p. 40-50.

69. Roe, A.D., et al., *Multilocus species identification and fungal DNA barcoding: insights from blue stain fungal symbionts of the mountain pine beetle.* Molecular Ecology Resources, 2010. **10**(6): p. 946-959.

70. Lindner, D.L. and M.T. Banik, *Intragenomic variation in the ITS rDNA region obscures phylogenetic relationships and inflates estimates of operational taxonomic units in genus Laetiporus.* Mycologia, 2011. **103**(4): p. 731-740.

Table 1. Collection location information for WPB populations sampled for their mycangial fungi.

Collection method	Date collected	Location	Elevation (m)	Nearest town	Identifier
Funnel	5/23/2011	34° 44′ N, 120° 44′ W	608	Placerville, CA	PL
Funnel	6/20/2011	34° 32′ N, 112° 32′ W	1,886	Prescott, AZ	PR
Funnel	6/21/2011	35° 10′ N, 111° 45′ W	2,253	Flagstaff, AZ	FL
Reared from bark	6/18/2011	44° 53′ N, 115° 42′ W	1,253	Yellow Pine, ID	YE
Reared from bark	6/17/2011	46° 04′ N, 114° 14′ W	1,326	Darby, MT	DA
Reared from bark	6/18/2011	44° 06′ N, 115° 21′ W	1,296	Lowman, ID	LO
Funnel	6/23/2011	47° 45′ N, 120° 25′ W	684	Entiat, WA	EN
Funnel	6/21/2011	48° 16′ N, 120° 11′ W	760	Twisp, WA	TW
Funnel and bark	6/22–8/31/2011	34° 10′ N, 116° 55′ W	1,908	San Bernardino, CA	SB
Funnel	6/26/2011	46° 53′ N, 113° 28′ W	1,311	Greenough, MT	LF
Reared from bark	6/23/2011	47° 16′ N, 117° 34′ W	640	Cheney, WA	CH
Funnel	7/4/2011	48° 34′ N, 118° 18′ W	764	Kettle Falls, WA	KE
Funnel and bark	7/2/2011	49° 41′ N, 119° 46′ W	655	Summerland, BC	SU
Funnel	6/29/2011	32° 53′ N, 107° 45′ W	2,475	Gila, NM	GI
Funnel	6/26/2011	46° 49′ N, 114° 08′ W	1,382	Missoula, MT	MI
Funnel	7/15/2011	45° 19′ N, 118° 19′ W	1,079	La Grande, OR	LA
Funnel	7/13/2011	40° 41′ N, 121° 13′ W	1,709	Old Station, CA	OL
Funnel	7/13/2011	45° 54′ N, 120° 42′ W	682	Goldendale, WA	GO
Funnel	7/14/2011	42° 46′ N, 121° 44′ W	1,415	Chiloquin, OR	CQ
Reared from bark	7/19/2011	44° 27′ N, 120° 26′ W	1,317	Prineville, OR	PV
Funnel	8/1/2011	37° 35′ N, 112° 15′ W	2,439	Tropic, UT	UT
Funnel	8/3/2011	36° 18′ N, 115° 40′ W	2,569	Mt. Charleston, NV	MTC
Funnel	8/5/2011	33° 28′ N, 109° 22′ W	2,215	Morenci, AZ	AP
New attack	8/8/2011	33° 28′ N, 105° 44′ W	2,383	Ruidoso, NM	RO
Funnel	8/10/2011	37° 21′ N, 107° 19′ W	2,229	Piedra, CO	PA
Funnel	8/19/2011	37° 59″ N, 120° 05′ W	1,375	Tuolumne City, CA	SierraI
Funnel	8/25/2011	38° 13′ N, 120° 22′ W	1,075	Avery, CA	SierraII
Funnel	9/8/2011	37° 02′ N, 119° 14′ W	1,017	Bretz Mill, CA	SierraIII
Funnel	9/1/2011	41° 17′ N, 122° 03′ W	1,098	McCloud, CA	MC

Table 2. Sequenced isolates of *Entomocorticium* and *Ceratocystiopsis* and their GenBank and CBS accession numbers.

Genus	Species	Isolate	Representative isolate[a]	Haplotype	Region sequenced	GenBank accession number	CBS accession number
Ceratocystiopsis	*brevicomi*	OL20	OL20	1	beta-tubulin	KJ620527	CBS137841
	brevicomi	CH11, FL27, PL02, SB06, KE15, LA17, MI18, MTC05, RO16, TW14, YE24, MC10, LF20, LO20, SierraII05, UT14, SU27, Sierra02	FL27	2	beta-tubulin	KJ620528	CBS137843
	brevicomi	PV13, DA23	PV13	3	beta-tubulin	KJ620529	CBS137844
	brevicomi	GO05	GO05	4	beta-tubulin	KJ620530	CBS137842
	brevicomi	CQ05	CQ05	5	beta-tubulin	KJ620531	CBS137840
Entomocorticium	B	CH06, EN05, GO06, TW12, CQ11, DA17, LF21, MC16, SierraIII13, KE22, LO18, OL13, SB13, YE18, PL01, SierraII2, MI22, LA6, PV11, PV19	SB13	A	ITS	KJ620521	CBS137846
	B	SU08, Sierra04, LA20, LA15, LA4, LA10, PV7, PV12, PV17, MI21, MI11, MI14, MI17, DA10, DA18	LA20	B	ITS	KJ620520	CBS137848
	B	AP14, UT12, RO10, GI04, FL19, PA12, MTC02, PR07	FL19	C	ITS	KJ620518	CBS137845
	B	B1037 (Hsiau and Harrington 2003)	B1037	A	ITS	KJ620519	CBS137851
	H	B1065 (Hsiau and Harrington 2003)	B1065		ITS	KJ620522	CBS137852
	A	APFr4	APFr4		ITS	KJ620523	CBS137849
	A	FLFr2	FLFr2		ITS	KJ620524	CBS137853
	C	B896 (Hsiau and Harrington 2003)	B896		ITS	KJ620525	CBS137850
	G	B1069 (Hsiau and Harrington 2003)	B1069		ITS	KJ620526	CBS137847

[a] Isolate deposited with CBS

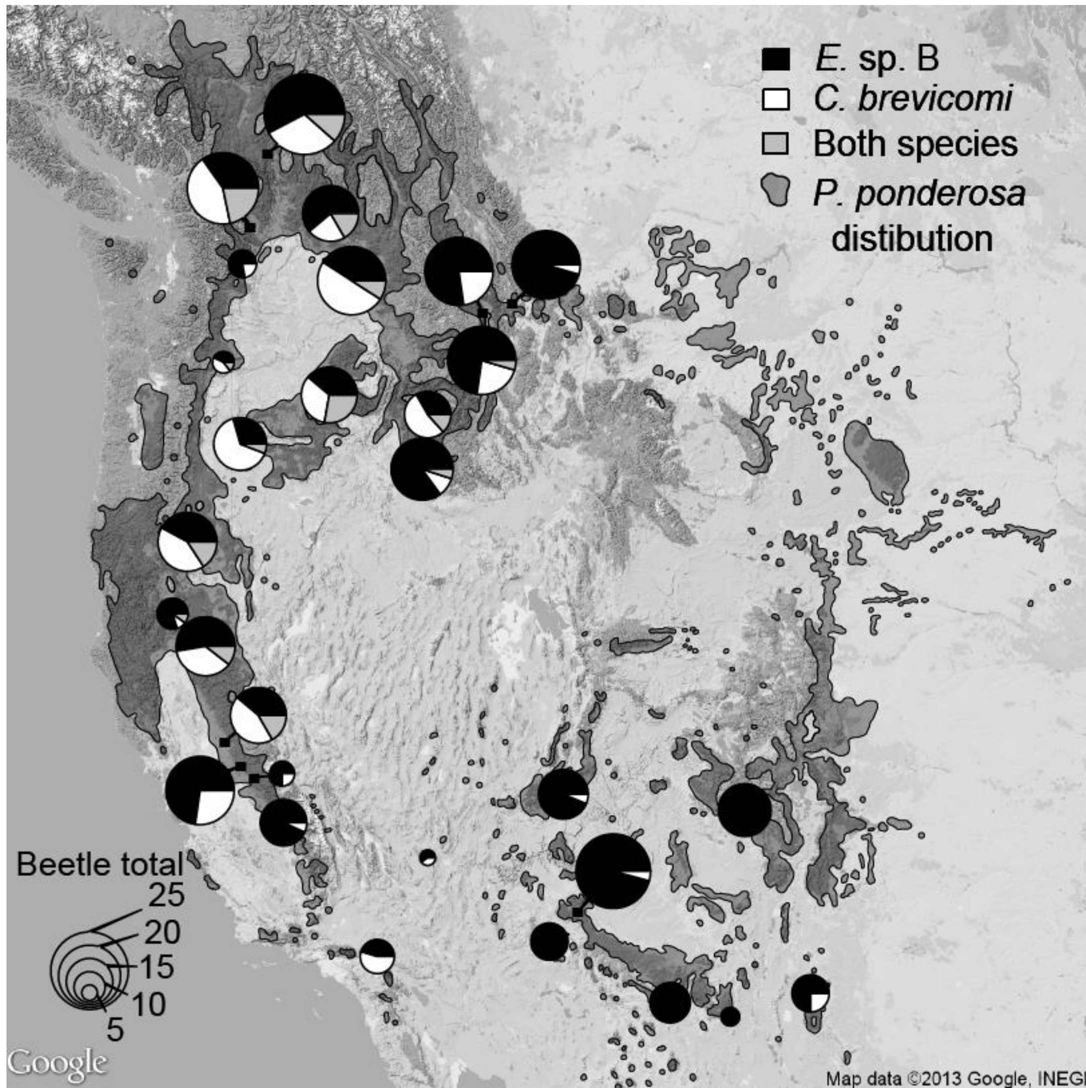


Figure 1. The relative frequency of the mycangial fungi of the western pine beetle (*Dendroctonus brevicomis*) from 29 collection areas in western USA. The distribution of the beetle generally follows the distribution of its primary host tree *Pinus ponderosa* (shown), except in northern CO, Wyoming, South Dakota, Nebraska, and eastern Montana, where it is absent.

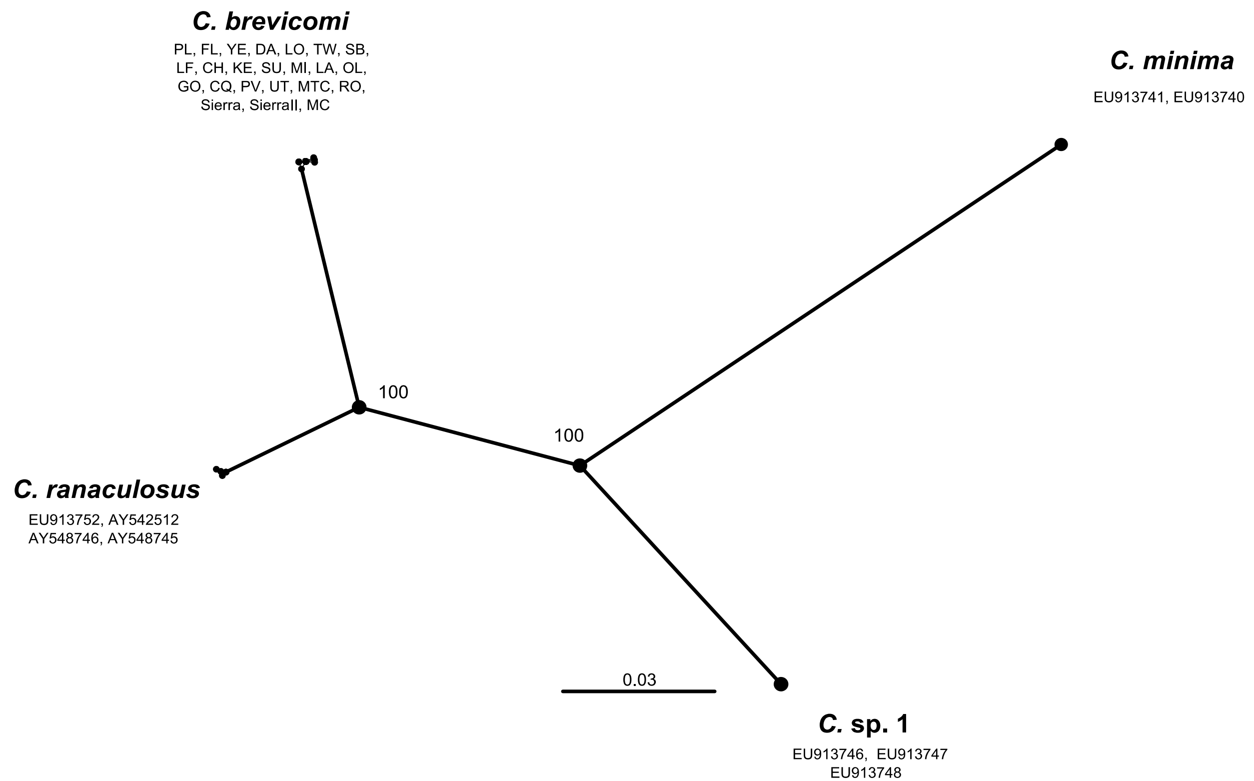


Figure 2. Unrooted phylogenetic tree for 23 *Ceratocystiopsis* isolates from geographically distinct WPB collection locations along with three closely related fungal species identified by BLAST searches. The RAxML analysis identified 102 alignment patterns in the substitution matrix which were used to construct the tree. GenBank accession number or collection point identifiers (Table 1) are provided for each isolate included in the analysis. Bootstrap values are included at nodes when > 50.

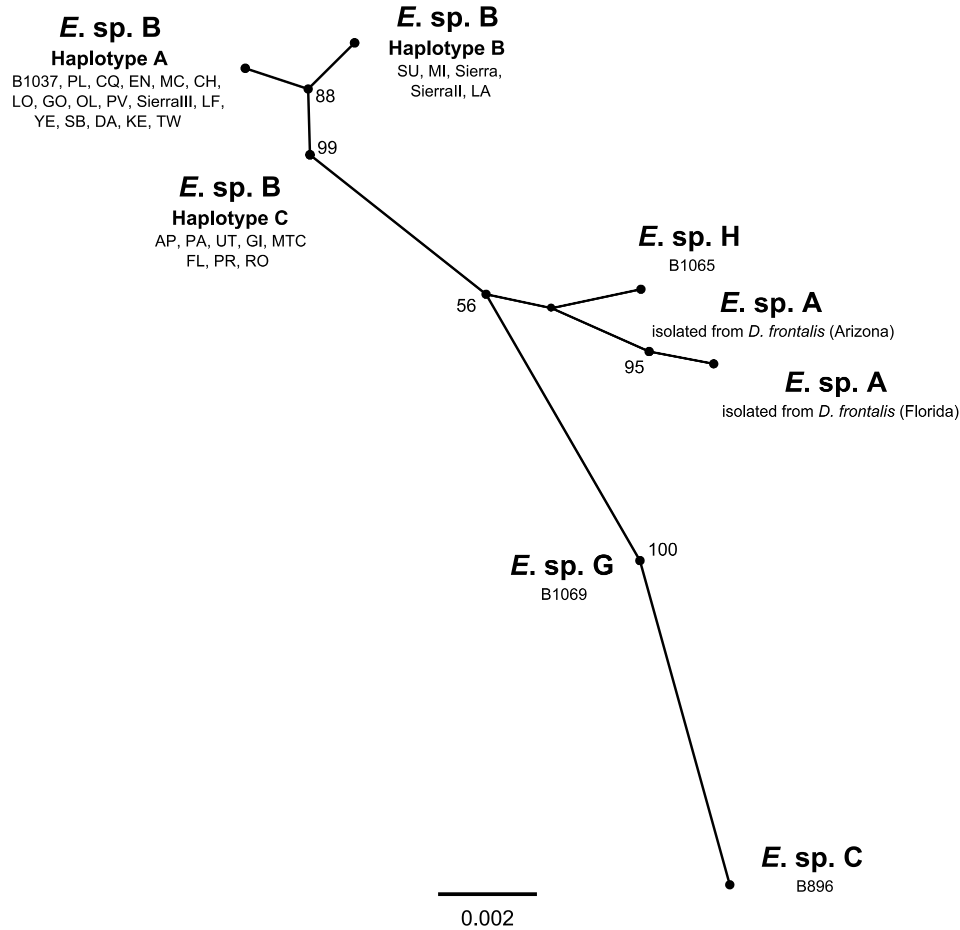


Figure 3. Unrooted phylogenetic tree for 29 *Entomocorticium* isolates from geographically distinct WPB collection locations along with reference isolates for closely related fungal species. RAxML analysis identified 41 distinct alignment patterns in the substitution matrix which were used to construct the tree. GenBank accession number or collection point identifiers (Table 1) are provided for each isolate included in the analysis. Bootstrap values are included at nodes when > 50.

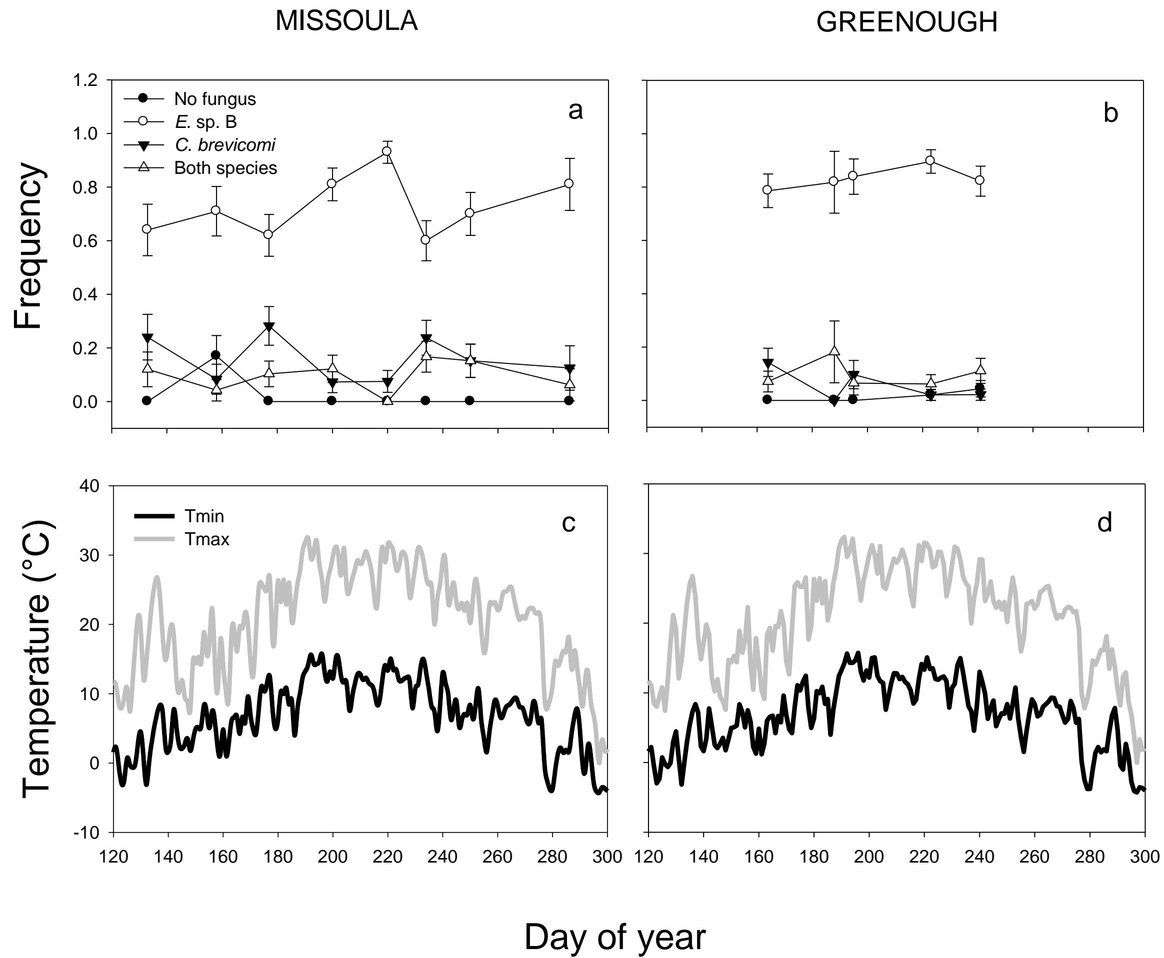


Figure 4. The frequency of western pine beetle (*Dendroctonus brevicomis*) carrying exclusively *Entomocorticium* sp. B, exclusively *Ceratocystiopsis brevicomi*, both species, or no fungi. Two sites, a) Missoula, and b) Greenough (Montana, USA), were sampled at multiple time points during the 2012 *D. brevicomis* flight season. Daily minimum (Tmin) and maximum (Tmax) temperatures are shown for the c) Missoula, and d) Greenough site*s*.

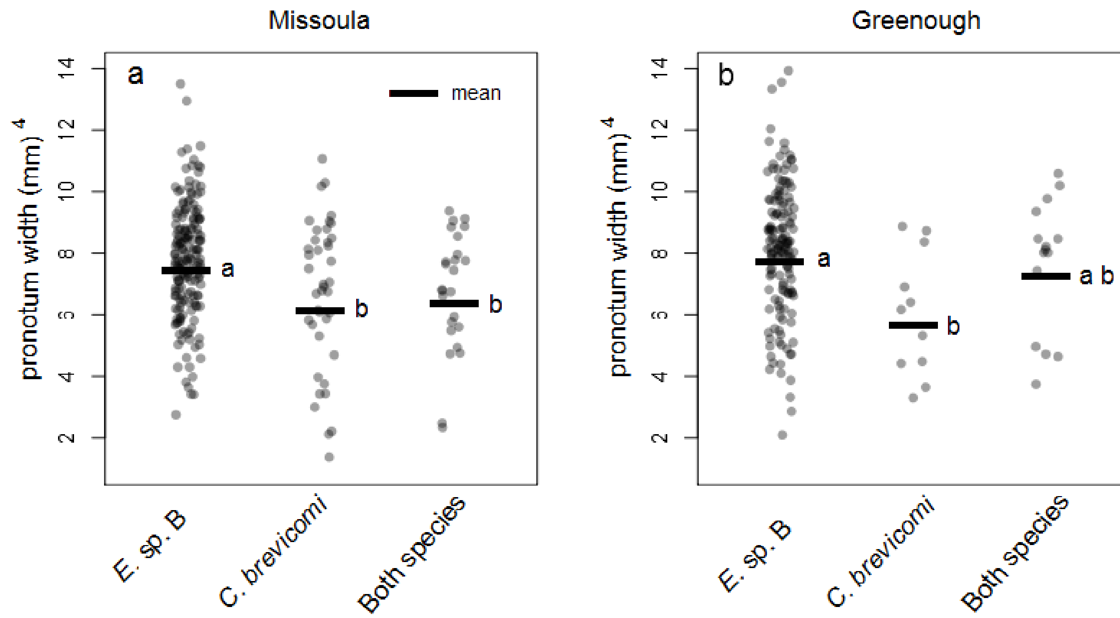
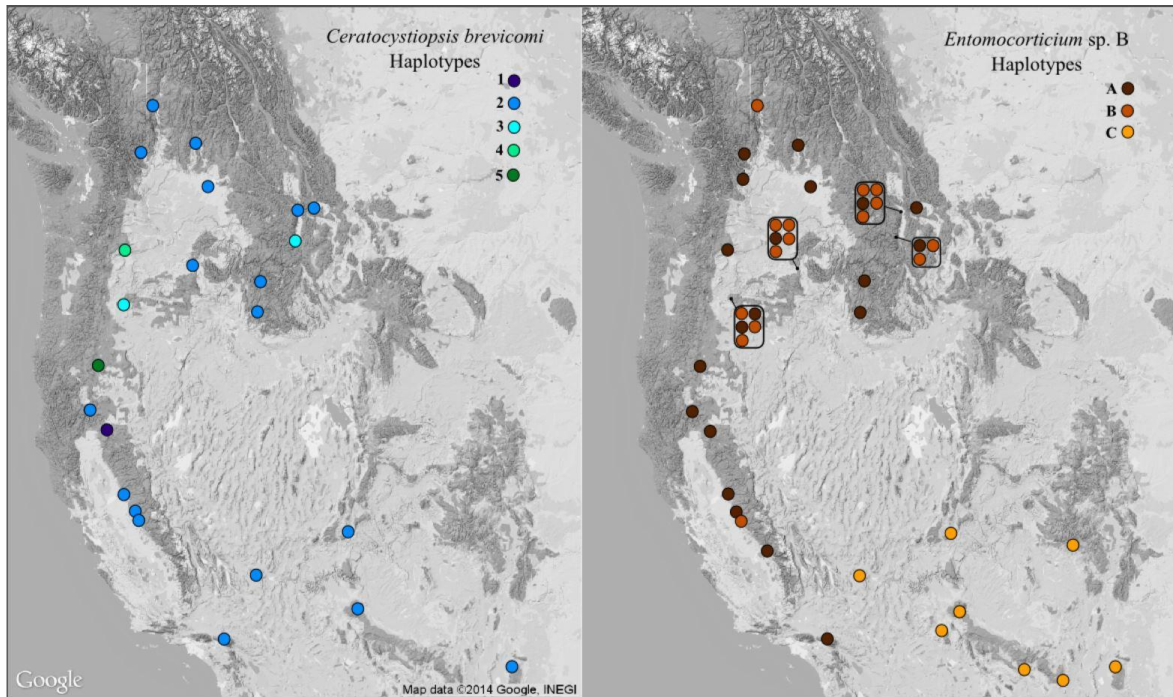


Figure 5. Comparisons of body size (pronotum width) of *Dendroctonus brevicomis* from two sites, a) Missoula, b) Greenough (Montana, USA) found carrying either *Entomocorticium* sp. B, *Ceratocystiopsis brevicomi*, or both fungi. Means within the same site with the same letter are not significantly different from one another (Tukey HSD tests, $p > 0.05$)



Supplementary file 1. Haplotype distributions for *Entomocorticium* sp. B and *Ceratocystiopsis brevicomi isolates*.

CHAPTER 2

Experimental evidence of bark beetle adaptation to a fungal symbiont

Bracewell, R.R. and D.L. Six, *Experimental evidence of bark beetle adaptation to a fungal symbiont.* Ecology and Evolution, 2015. **5**(21): p. 5109-5119.

ABSTRACT

The importance of symbiotic microbes to insects cannot be overstated; however, we have a poor understanding of the evolutionary processes that shape most insect-microbe interactions. Many bark beetle (Coleoptera: Curculionidae, Scolytinae) species are involved in what have been described as obligate mutualisms with symbiotic fungi. Beetles benefit through supplementing their nutrient poor diet with fungi and the fungi benefit through gaining transportation to resources. However, only a few beetle-fungal symbioses have been experimentally manipulated to test if the relationship is obligate. Furthermore, none have tested for adaptation of beetles to their specific symbionts, one of the requirements for coevolution. We experimentally manipulated the western pine beetle-fungus symbiosis to determine whether the beetle is obligately dependent upon fungi and to test for fine-scale adaptation of the beetle to one of its symbiotic fungi, *Entomocorticium* sp. B. We reared beetles from a single population with either a natal isolate of *E.* sp. B (isolated from the same population from which the beetles originated), a non-natal isolate (a genetically divergent isolate from a geographically distant beetle population), or with no fungi. We found that fungi were crucial for the successful development of western pine beetles. We also found no significant difference in the effects of the natal and non-natal isolate on beetle fitness parameters. However, brood adult beetles failed to incorporate the non-natal fungus into their fungal transport structure (mycangium) indicating adaption by the beetle to particular genotypes of symbiotic fungi. Our results suggest that beetle-fungus

mutualisms and symbiont fidelity may be maintained via an undescribed recognition mechanism of the beetles for particular symbionts that may promote particular associations through time.

INTRODUCTION

Many insects are involved in symbiotic associations with microbes that provide nutrition crucial for insect survival [1, 2]. In obligate endosymbioses where the symbiont is transferred vertically from parent to offspring, both theoretical and empirical studies have demonstrated the relative ease at which coevolution (reciprocal adaptation) and co-cladogenesis can occur [3-5]. However, a large number of insect-microbe symbioses, and particularly insect-fungal symbioses, are ectosymbioses, where symbiont transfer can be imperfectly vertical or even horizontal [2]. In such systems, coevolution and/or co-cladogenesis has been regarded as less likely to occur due to the potential for swapping and invasion. Despite this assumption, numerous studies have now observed strong fidelity among hosts and symbionts in several ectosymbioses [6-8]. However, the mechanisms that maintain fidelity as well as the occurrence of coevolution remain severely understudied in most ectosymbiotic systems.

Bark beetles (Coleoptera: Curculionidae, Scolytinae) are some of the most ecologically and economically important forest insects and many are involved in tightly linked ectosymbioses with fungi [9-11]. These symbioses remain understudied in many important aspects including how dependent the partners are upon one another, and whether hosts and symbionts exhibit coevolution or codiversification [11, 12]. Some bark beetle-symbioses exhibit characteristics that imply coevolution. A number of bark beetle species have evolved specialized exoskeletal structures, called mycangia, that aid in transporting their fungal symbionts between host trees. The fungal symbionts also exhibit adaptations to their hosts including the production of sticky

spores that are specialized for insect transport [13-15]. These symbioses are generally considered mutualisms because the mycangial fungi gain transportation to host trees while, in return, the fungi provide nutritional benefits to the developing insect [16-18]. Most mycangium-bearing bark beetle–fungal symbioses are also considered obligate, although very few have been experimentally tested in this regard. In part, this has been due to the difficulty of experimentally manipulating the symbiosis.

The western pine beetle (*Dendroctonus brevicomis* LeConte) symbiosis is a powerful system to address questions of coevolution and codiversification in ectosymbioses, in general, and beetle-fungus symbioses, in particular. This symbiosis, involves a beetle with two symbiotic fungal partners, *Entomocorticium* sp. B (Basidiomycota) and *Ceratocystiopsis brevicomi* (Ascomycota) [15, 19-21], that show remarkable fidelity with their host across its entire range [22]. *Entomocorticium* sp. B and *C. brevicomi* have also never been found outside of the western pine beetle symbiosis. The two fungi are carried in a prothoracic mycangium found only in females. During tree colonization, they inoculate the tree with their symbiotic fungi and oviposit in the tree's phloem layer. Larval feeding and development initially occurs in the phloem where the developing larvae feed on a combination of fungi and phloem. However, at about the second instar, larvae transition from the more nutrient rich phloem to the nutrient poor bark [23](Fig. 1a,b). This transition is hypothesized to be mediated by the symbiotic fungi, of which one species, *E*. sp. B, may be particularly important given that *Entomocorticium* species are cellulolytic and can grow not just on phloem but also on bark [24].

The western pine beetle is restricted to ponderosa pine (*Pinus ponderosa* Douglas) across most of its range. Genetic evidence indicates the beetle is actually two cryptic species that are geographically isolated on two subspecies of ponderosa pine [25]. Mitochondrial DNA sequence

divergence suggests these cryptic species of beetle have been isolated for a few million years [25]. Palynological and molecular data for the tree suggest that the tree subspecies formed in glacial refugia during the Pleistocene [26-29]. There is also evidence of genetic divergence within *E*. sp. B that corresponds to patterns of divergence in both the host tree and insect [22]. Three distinct haplotypes of *E*. sp. B have been identified, and haplotype A and B co-occur and are found only in beetle populations in the westernmost portion of the distribution (CA, OR, WA, ID, MT and BC, Canada), while haplotype C occurs exclusively in the southwestern U.S. (CO, UT, AZ, NV, NM).

A powerful way to test for dependency and adaptation is to conduct symbiont removals and experimental swapping of symbionts [7, 30]. Although the western pine beetle-fungus symbiosis has been described as an obligate mutualism, to date there have been no manipulative experiments to test this hypothesis, nor whether adaptation of the beetle to particular fungi has occurred. Here, we focus our investigation on one symbiont of this beetle, *E*. sp. B, since this partner is thought to be the superior symbiont in this system for supporting beetle nutrition. Adult beetles captured carrying *E*. sp. B tend to be, on average, larger than those developing with *C. brevicomi*, suggesting that developing larvae gain more nutrition while feeding on this fungus [22]. *Entomocorticium* sp. B is also more prevalent than *C. brevicomi*, indicating it may play a dominant role in the symbiosis. Further, the genetic differences found between *E*. sp. B haplotypes indicate that phenotypic differences may occur among the haplotypes that could alter the symbiosis.

The objectives of this study were to determine if 1) mutualistic fungi, particularly *E*. sp. B, are crucial for western pine beetle development, and 2) if we could detect evidence of adaptation by the beetle to specific isolates of *E*. sp. B. We did this by rearing beetles from one

cryptic species with their normal haplotype of *E.* sp. B (haplotype A, designated here as natal), with a haplotype associated with the other cryptic beetle species (haplotype C, designated here as non-natal,), or with no fungi. We then characterized the effect of these three treatments on beetle development and fitness.

METHODS

Generating aposymbiotic adult beetles

Live western pine beetles were collected near Missoula MT (46° 49′ N, 114° 08′ W) in June 2012 using Lindgren funnel traps baited with chemical attractants (Synergy Semiochemical Corp., Burnaby, BC, Canada; part P130, western pine beetle trap lure). All adult beetles were surface sterilized (30s in 70% EtOH) to remove potentially antagonistic fungi which can be carried externally and hamper lab rearing. Sex was then determined by the presence of the mycangial swelling on the pronotum of females and tubercles on the frons of males [31]. Males and females were then paired in sections of a freshly cut ponderosa pine felled at The University of Montana Lubrecht Experimental Forest (46° 53′ N, 113° 28′ W). Detailed methods for rearing bark beetles are described elsewhere [32]. Specific to this study, a total of 15 sections of ponderosa pine (~ 33 cm in length) were each infested with 10-13 beetle pairs. To produce large numbers of pupae, tree sections containing beetles were stored at room temperature (~21°C) for 40 d allowing most to reach the pupal stage [23]. Pupae were collected from the sections by removing the bark which was then fractured to expose the pupal chambers. Pupae were then removed and placed in Petri dishes lined with filter paper moistened with distilled water.

Larvae void their guts prior to pupation but may still carry microbes including fungi on their exoskeletons. Therefore, we surface sterilized the pupae using a series of three short EtOH

washes conducted over three days. Washes consisted of placing pupae for 10s in 70% EtOH, before quickly dipping them in distilled water and transferring them to a Petri dish. We then placed surface-sterilized pupae into pseudo-pupal chambers constructed from fresh ponderosa pine bark (Fig. 1c). To mimic a western pine beetle pupal chamber, 8 x 8 x 3 cm pieces of ponderosa pine bark were cut from a tree and a small holes were drilled into the bark piece (Fig. 1c). To maintain humidity for the developing insect, bark pieces containing pupae were placed into plastic containers floating in a bath of distilled water in air-tight rearing containers. The rearing containers were maintained at room temperature (~21°C) and pupae allowed to develop into adults.

Approximately 350 pupae were placed into pseudo-pupal chambers. Rearing containers were checked daily and any eclosed and putatively 'fungus free' adults were collected and placed into sterile Petri dishes and held at ~4° C. Due to the difficulties of manipulating small insects during a sensitive life stage, a large number of pupae were processed to ensure enough individuals survived for use in the experiment. To confirm that adult beetles did not have fungi in their mycangia following rearing and surface sterilization, we attempted to isolate fungi from the mycangia of nine females (14% of all females), all of which were negative for fungi. Methods used to isolate mycangial fungi from western pine beetles are detailed in Bracewell and Six [22].

Propagating fungal symbionts

Two isolates of fungi were used in this study. The natal isolate (MI22) was originally isolated from a beetle collected near Missoula MT (46° 49′ N, 114° 08′ W). The non-natal isolate (RO10), was originally isolated from an individual of the other cryptic western pine beetle

species collected near Ruidoso NM (33° 28′ N, 105° 44′ W) [22]. Both isolates have typical morphology and display the same growth patterns on MEA of the fungal populations from which they were isolated [22]. Although they are both currently considered *E.* sp. B, they are genetically divergent; MI22 has been identified as haplotype A and RO10 as haplotype C [22]. Pairwise distance between A and C haplotypes (p-distance) is 0.004 over the ITS2-LSU region [22]. Haplotype A and Haplotype C are also visually distinct when grown on 2% MEA (Fig. 1d). The isolates were grown on 2% MEA for ~ 3 weeks prior to experimental manipulation to ensure the mycelia were in the active growth stage.

Manipulating the symbiosis

One ponderosa pine was felled at Lubrecht Experimental Forest (46° 53′ N, 113° 28′ W) in August 2012. The tree was first cut crosswise into 33 cm sections. Then each section was quartered lengthwise resulting in a total of 198 cm^2 of phloem/bark for each beetle pair per replicate. Tree sections were coated in paraffin wax along the four cut edges to help maintain natural levels of moisture.

Due to slight differences in phloem thickness among sections which could influence total brood production, treatments (natal, non-natal, and no fungus) were randomly assigned to the tree sections. 17 tree sections (replicates) per treatment were assigned to each of the three treatments at the start of the experiment. To establish the fungus in a section, one 4 mm diameter plug of agar was taken from an MEA plate containing either the natal or the non-natal fungus. No fungus treatments received a plug of MEA. Each plug was smeared inside a hole drilled into the phloem layer at the base of the tree section. Trials conducted prior to our experiment indicated that seven days was adequate for both the natal and non-natal fungus to establish in the

tree phloem. Therefore, after inserting the agar plug, all tree sections were held for seven days. Surface sterilized female/male pairs were then inserted into the same drill hole (female first) and a piece of wire screen was fixed over the hole to prevent escape. Each tree section was then placed in a rearing container and monitored daily for emergence of offspring. Tree sections were maintained at room temperature (~21°C) under natural light conditions.

To assess the effect of a particular treatment on brood production and the resulting fitness of offspring we recorded when each brood beetle emerged, their sex, and their pronotum width. Pronotum width is a proxy measure for overall beetle size [33] and size is positively correlated with offspring production [34]. To estimate the pronotum width, digital images were taken using a Leica EZ4D stereomicroscope with built-in 3-megapixel camera. To confirm fungal growth in the tree sections, we visually inspected all replicates after completion of the experiment. To confirm we were able to re-establish the symbiosis by inoculating the tree sections with fungal isolates, we isolated fungi from the mycangia of a subset of brood females from each replicate soon after they emerged into the rearing containers. We also confirmed which fungal isolate (MI22 or RO10) was recovered from each mycangium by sequencing the ITS2-LSU region using the primer pair ITS3 and LR3 [35, 36]. Methods used to isolate the mycangial fungi, and to perform PCR and DNA sequencing are described in Bracewell and Six [22]. Sequences were compared to reference sequences for MI22 and RO10 (Genbank accessions KJ620521 and KJ620518).

To further investigate the effects of the three treatments on western pine beetle brood production, development and fitness, parent and larval tunnels were measured after brood emergence was complete. We traced the parent galleries and all larval tunnels on transparency sheets (Fig 1e). Parent gallery length was estimated as the sum of all tunnels created by parent

beetles per section. Due to the network of tunnels created by larvae, and the state of decay of the samples after completion of the experiment, it was impossible to estimate the number of larvae or egg niches produced by each beetle pair. However, we could measure total larval tunnel length which allowed us to determine whether larvae from the three treatments fed differentially in the phloem layer (an indication of differential nutrient availability due to the presence/absence of symbiotic fungi). Here, total larval tunnel length was calculated for each replicate as the sum of larval tunnel distances divided by the total gallery length of the parents. In other studies, a positive correlation has been found between parent gallery length and reproductive output [37, 38]. Therefore, this metric provides an estimate of larval feeding differences that takes into account the different quantities of larvae found in galleries of differing lengths.

Statistical analysis

All analyses were conducted using the statistical package R v. 3.1.2 (R Development Core Team, 2011). To investigate the effect of the fungal treatments on development time and on the size of brood beetles, and because both development time and beetle size were found to be normally distributed, we fit linear mixed models (LMMs) using the *nlme* package in R [39]. For beetle size comparisons, we analyzed the male and female data separately, since females are on average larger than males [40]. We treated each replicate as a random effect in the model to account for non-independence of brood beetles within replicates. *Post hoc* pairwise comparisons between the three treatments were performed using Tukey's HSD (Honestly Significance Difference) tests in the R *multcomp* package [41]. To test for the influence of fungal treatment on total length of parent gallery, our standardized total larval tunneling length measure, number of offspring, and proportion of brood that were female, we fit generalized linear models (GLMs)

with the *glm* package in R and specified appropriate error distributions for each response variable. Significance of the fixed effects in the model were determined using Wald chi-square tests and pairwise comparisons between treatments were performed using Tukey's HSD tests. Total larval tunnel lengths and length of parent galleries were found to be normally distributed and were modeled with Gaussian distributions. The number of offspring were count data and so were modeled using a poisson distribution. The proportion of females produced was found to be overdispersed (more variance than expected) and thus modeled using a quasi-binomial distribution. For all models, adequate model fit was determined by evaluating the residual deviance.

RESULTS

The experiment ran for 312 days and was stopped when no brood beetles emerged from any replicate for > 14 days. At the completion of the experiment, 14, 12, and 13 replicates per treatment (natal, non-natal, and no fungus, respectively) were considered for analyses. Criteria for inclusion in analysis were that parent gallery length was > 0 cm (indicating successful pairing and tunneling by the parents).

A total of 742 brood adult beetles were recovered from rearing containers. A subset of brood females from each replicate (when present) were used to isolate fungi from their mycangia to confirm that experimentally manipulated fungi were successfully transferred to the gallery of the insect and subsequently acquired in brood adult mycangia. We isolated fungi from the mycangia of 1 to 14 beetles from all replicates that produced females and in total isolated fungi from 132 individuals (Appendix 1). Replicates where we were unable to attempt fungal isolation from a female (n = 3 natal, 11 no fungus, 2 non-natal) were kept in their respective treatment

category. We sequenced the ITS2-LSU regions of three isolates from the mycangia of beetles from three replicates each of the natal fungus treatment and the non-natal fungus treatment (only three replicates had fungi), and one isolate from the only replicate of the no fungus treatment that was positive for mycangial fungi. All of the isolates possessed DNA sequences identical to haplotype A, indicating that there were instances in the non-natal and no fungus treatments where surface sterilization of the pupae was unsuccessful After removing these replicates from further analyses, we were left with, 14, 9, and 12 replicates per treatment (natal, non-natal, and no fungus, respectively). For the natal fungus treatment, we recovered natal fungi from 73% of replicates (8 of 11 replicates, 31 of 49 beetles, mean = 4 females isolated per replicate). In contrast, we were unable to recover the non-natal fungus from a single mycangium of brood females (0 of 7 replicates, 0 of 65 beetles, mean = 8 females isolated per replicate) (χ^2 (1, N = 114) = 56.48, $p < 0.0001$). All tree sections in the natal and non-natal treatments, showed evidence of *E*. sp. B growth in the phloem in both the parent gallery and larval tunnels.

We found no significant differences in parent gallery length among the three treatments (Table 1,Table 2) suggesting that in terms of gallery construction, the parent beetles were not affected. There was evidence of larval tunnels (and therefore, oviposition, egg hatch, and larval feeding), in all treatments, and no significant differences were found in the amount of larval tunneling among treatments (Table 1, Table 2). Although there was no evidence of a decrease in the level of reproductive input from parent adults, or alteration in tunneling distance by larvae, there was a highly significant difference in the total number of offspring produced across treatments (Table 1, Table 2). There was a near absence of adult offspring in the no fungus treatment, while the natal and non-natal treatments produced adult offspring but did not differ significantly in number (Fig. 2). Examination of larval tunnels in the no fungus treatment

indicated that nearly all larvae perished prior to tunneling into the bark. The proportion females produced was not statistically different between the natal and non-natal treatments (Table 1, Table 2).

When comparing the relative size of brood from the natal and non-natal treatments, and with respect to brood females, we did not find a relationship between adult size and development time ($F_{1,209} = 0.330$, $p = 0.567$). There was also no effect of fungal treatment ($F_{1,16} = 0.372$, $p = 0.550$) (Fig. 3a), and no interaction between development time and fungal treatment ($F_{1,209} = 2.029$, $p = 0.156$). Results from size comparisons of brood males was similar to that of females. There was no significant relationship between the size of male beetles and their development time ($F_{1,264} = 1.015$, $p = 0.315$), nor did fungal treatment affect adult size ($F_{1,18} = 0.372$, $p = 0.550$) (Fig. 3b). There was no interaction between development time and the fungal treatment ($F_{1,264} = 0.516$, $p = 0.473$).

Development time of brood beetles was highly variable (GLM model parameter estimate for development time of natal beetles = 151.49 (±15.15) days and non-natal beetles = 168.15 (±23.68) days). There was no significant difference in development times of either sex ($F_{1,522} = 0.060$, $p = 0.807$). Fungal treatment (natal or non-natal) also did not significantly influence development time ($F_{1,19} = 0.332$, $p = 0.571$), and there was no interaction between the development time of either sex and fungal treatment ($F_{1,522} = 0.293$, $p = 0.589$).

DISCUSSION

Many insects rely on mutualistic symbionts for nutritional supplementation [1, 2]. Experimentally testing the obligate nature of the mutualism is an important first step in understanding these types of symbiosis. Further, verifying the level of reciprocal adaptation

between the host and symbiont is crucial for establishing the strength of coevolution between species [42]. Here, we demonstrated a requirement of symbiotic fungi, specifically *E*. sp. B, for supporting growth and development of western pine beetle. We also found that the fungal isolate/haplotype obtained from western pine beetle from the southwest (i.e., the other cryptic beetle species) was capable of supporting development of beetles from Montana. However, our most striking finding was the inability of brood adult beetles from Montana to acquire the southwest isolate/haplotype in their mycangia. Our results suggest that western pine beetles are adapted to particular fungal partners and that fidelity may be enforced through mycangial selectivity.

We observed a near complete loss of brood production when beetles were reared without a fungal symbiont. Our results are similar to studies that have been conducted on the closely related southern pine beetle (*Dendroctonus frontalis* Zimmerman) system, where there is a strong negative effect on beetles when they are reared without their mycangial fungi (*Entomocorticium* sp. A and *C. ranaculosus*). Barras [43] experimentally manipulated the southern pine beetle symbiosis and found that non-manipulated adult beetles produced nearly triple (2.93×) the number of adult offspring compared to beetles that were surface sterilized as pupae. Goldhammer et al. [44] conducted laboratory experiments with beetles naturally with and without mycangial fungi, and found that beetles without mycangial fungi were able to produce brood adults. Unfortunately, the number of offspring per pairing was not quantified in the experiment. Goldhammer et al. [44] did find that brood adults that developed without fungi were much smaller, unable to reproduce, and unsuccessful at initiating galleries. Our methods for testing the importance of the mycangial fungi differed from those of Barras [43] and Goldhammer et al. [44] in that we subjected all pupae to surface sterilization and then

reestablished the symbiosis with only one symbiont. Our methods, thereby, isolated the effect of only one factor, *E*. sp. B, on beetle reproduction and eliminated surface sterilization as a confounding factor. In both Barras [43] and Goldhammer et al. [44], yeasts and bacteria remaining on the exoskeleton or in the mycangia may have influenced results.

In our study, we found no evidence of a reduction in parent gallery length or of reduced oviposition in our no fungus treatment, suggesting little direct impact on reproductive investment from aposymbiotic parent adults. Our results are consistent with Barras [43] who also failed to find differences in reproductive input from surface-sterilized southern pine beetles. However, our results and those of Barras [43] are in contrast to Goldhammer et al.[44], who identified decreases in gallery production and oviposition of southern pine beetles without mycangial fungi relative to those with fungi. The differences between the studies warrant further research into the relative importance of the mycangial fungi to different bark beetle life stages.

Comparisons of brood production of beetles reared on the two different haplotypes of *E*. sp. B (i.e., natal and non-natal) in our study suggest that both are equally capable of providing the nutritional requirements of the host beetle. We observed no differences between natal and non-natal treatments with regard to total number of brood adults, their size, or their development time, despite the fact that the two fungal isolates are genetically divergent [22] and may have been geographically isolated, along with their hosts, for a long period of time [25]. This may indicate strong selection to maintain characteristics in the fungi that provide appropriate nutrients to the host and that these requirements do not differ between the two cryptic species of beetle. This may be a general feature of mycangial fungi as a fungal swap experiment in ambrosia beetles (bark beetles that are dependent on mycangial fungi) also suggests little impact on reproductive output when beetles were reared with a different mycangial fungus [45].

However, while the fungi did not differ in their effects on beetle development and productivity, the Montana beetles never acquired the southwestern fungus in their mycangia and the resulting brood females were aposymbiotic. This implies that beetles may have diverged along with their fungi and that they are adapted to particular genotypes of symbionts enforcing a high degree of specificity and fidelity at a very fine scale. Although our experimental design left us unable to quantify fungal growth in each treatment, or confirm fungal sporulation of the non-natal isolate in the pupal chamber (necessary for incorporation in the mycangia), our results do not suggest that the non-natal fungus grew or sporulated differently than the natal fungus. Had the non-natal fungus grew poorly or not sporulated, brood adults would have likely been smaller (e.g., [44]) and development times would have likely been significantly different between treatments. Many bark beetle species maturation feed on fungal spores in the pupal chamber and fungal absence can lead to delayed emergence. It is important to note that due to limitations of our experiment, only one representative isolate per haplotype was tested. Although our results likely apply broadly to each fungus, we cannot rule out isolate specific effects influencing our results. Future work should examine isolate specific differences and how they might impact the symbiosis in general as recent work suggests some variation in growth rate of *Entomocorticium* isolates [46]. Further, an experimental manipulation of the western pine beetle symbiosis that tests if beetles from the southwest can incorporate haplotype A into their mycangia will help determine if our observed pattern of specificity is reciprocal.

There are a number of species in the bark beetle genus *Dendroctonus* that possess mycangia [47]. Many of these species overlap in distribution and in host tree species range [31] and are often found co-inhabiting the same tree. This means that, although symbiont transfer from parent to offspring is thought to primarily occur via vertical transfer, beetles that co-occur

in host trees are exposed to a large pool of potential fungal symbionts that occur due to the presence of congeneric beetles. Regardless of spatial and temporal overlap, these bark beetles still exhibit high fidelity with their fungal partners. Research to date suggests that, among mycangium-bearing *Dendroctonus*, there is remarkable fidelity of particular fungi with their host beetles and no evidence of swapping (although, in some cases, additional partners have been acquired) [8, 22, 48, 49].

How the beetle maintains these associations through time is not well understood, but our results indicate mycangia may play a key role enforcing specificity. Bark beetle mycangia have complex morphologies and contain glands which secrete substances thought to nurture fungi during transport [50-52]. Given the importance of these structures in maintaining the symbiosis through consistent dissemination between generations of beetles, it is plausible the structure and its glandular secretions may play a primary role in filtering fungal species and promoting associations with specific beneficial symbiotic partners. Future research aimed at identifying the substance(s) being excreted by the mycangia and additional fungal swapping experiments in other bark beetles will go a long way in understanding the ability of the mycangia to screen fungal symbionts and if glandular excretions may be fine-tuned to promote particular associations.

For insects involved in obligate mutualisms with microbes, maintaining associations with particular partners is crucial for the success and stability of the symbiosis. However, currently, we have only a rudimentary understanding of the mechanisms that underlie fidelity or evolutionary processes in ectosymbioses, including bark beetle-fungus symbioses. We have shown in other work that the western pine beetle symbiosis exhibits high fidelity [22]. In the research reported in this paper, we have shown through experimental manipulation of the

symbiosis, that fungi are critical to the beetle's survival. Further, our results suggests that the beetle's mycangia can distinguish between closely related fungal isolates and may play a key role in maintaining specificity. This, in turn, indicates adaptation of the beetle at a fine scale to its fungal partners. Additional research is needed to reveal whether fidelity and coevolution are common in bark beetle-fungus symbioses and what conditions facilitate or constrain coevolution and codiversification.

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REFERENCES

1. Moran, N.A., *Symbiosis as an adaptive process and source of phenotypic complexity.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**: p. 8627-8633.
2. Mueller, U.G., et al., *The evolution of agriculture in insects.* Annual Review of Ecology Evolution and Systematics, 2005. **36**: p. 563-595.
3. Conord, C., et al., *Long-term evolutionary stability of bacterial endosymbiosis in curculionoidea: Additional evidence of symbiont replacement in the dryophthoridae family.* Molecular Biology and Evolution, 2008. **25**(5): p. 859-868.

4. Clark, M.A., et al., *Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence.* Evolution, 2000. **54**(2): p. 517-525.
5. Moran, N.A. and G.M. Bennett, *The tiniest tiny genomes.* Annu Rev Microbiol, 2014. **68**: p. 195-215.
6. Mehdiabadi, N.J., et al., *Symbiont fidelity and the origin of species in fungus-growing ants.* Nature Communications, 2012. **3**.
7. Seal, J.N. and U.G. Mueller, *Instability of novel ant-fungal associations constrains horizontal exchange of fungal symbionts.* Evolutionary Ecology, 2014. **28**(1): p. 157-176.
8. Alamouti, S.M., et al., *Gene genealogies reveal cryptic species and host preferences for the pine fungal pathogen Grosmannia clavigera.* Mol Ecol, 2011. **20**(12): p. 2581-602.
9. Paine, T.D., K.F. Raffa, and T.C. Harrington, *Interactions among scolytid bark beetles, their associated fungi, and live host conifers.* Annual Review of Entomology, 1997. **42**: p. 179-206.
10. Harrington, T.C., *Ecology and evolution of mycophagous bark beetles and their fungal partners*, in *Ecological and Evolutionary Advances in Insect-Fungal Associations*, F.E. Vega and M. Blackwell, Editors. 2005, Oxford University Press. p. 257-291.
11. Six, D.L., *Ecological and evolutionary determinants of bark beetle—fungus symbioses.* Insects, 2012. **3**(1): p. 339-366.
12. Six, D.L. and T.D. Paine, *Phylogenetic comparison of ascomycete mycangial fungi and Dendroctonus bark beetles (Coleoptera : Scolytidae).* Annals of the Entomological Society of America, 1999. **92**(2): p. 159-166.

13. Upadhyay, H.P., *A monograph of Ceratocystis and Ceratocystiopsis*. 1981, Athens, Georgia: The University of Georgia Press.

14. Jacobs, K. and M.J. Wingfield, *Leptographium species: Tree pathogens, insect associates, and agents of blue-stain*. 2001, St. Paul, Minnesota: The American Phytopathological Society.

15. Hsiau, P.T.W. and T.C. Harrington, *Phylogenetics and adaptations of basidiomycetous fungi fed upon by bark beetles (Coleoptera : Scolytidae).* Symbiosis, 2003. **34**(2): p. 111-131.

16. Coppedge, B.R., F.M. Stephen, and G.W. Felton, *Variation in Female Southern Pine-Beetle Size and Lipid Content in Relation to Fungal Associates.* Canadian Entomologist, 1995. **127**(2): p. 145-154.

17. Ayres, M.P., et al., *Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi.* Ecology, 2000. **81**(8): p. 2198-2210.

18. Bleiker, K.P. and D.L. Six, *Dietary benefits of fungal associates to an eruptive herbivore: Potential implications of multiple associates on host population dynamics.* Environmental Entomology, 2007. **36**(6): p. 1384-1396.

19. Hsiau, P.T.W. and T.C. Harrington, *Ceratocystiopsis brevicomi sp nov, a mycangial fungus from Dendroctonus brevicomis (Coleoptera: Scolytidae).* Mycologia, 1997. **89**(4): p. 661-669.

20. Whitney, H.S. and F.W. Cobb, *Non-Staining Fungi Associated with Bark Beetle Dendroctonus Brevicomis (Coleoptera-Scolytidae) on Pinus Ponderosa.* Canadian Journal of Botany, 1972. **50**(9): p. 1943-&.

21. Paine, T.D. and M.C. Birch, *Acquisition and Maintenance of Mycangial Fungi by Dendroctonus-Brevicomis Leconte (Coleoptera, Scolytidae).* Environmental Entomology, 1983. **12**(5): p. 1384-1386.

22. Bracewell, R.R. and D.L. Six, *Broadscale Specificity in a Bark Beetle-Fungal Symbiosis: a Spatio-temporal Analysis of the Mycangial Fungi of the Western Pine Beetle.* Microbial Ecology, 2014. **68**(4): p. 859-870.

23. Miller, J.M. and F.P. Keen, *Biology and control of the western pine beetle: a summary of the first 50 years of research*. 1960, Washington, DC: U.S. Department of Agriculture.

24. Valiev, A., Z.B. Ogel, and K.D. Klepzig, *Analysis of cellulase and polyphenol oxidase production by southern pine beetle associated fungi.* Symbiosis, 2009. **49**(1): p. 37-42.

25. Kelley, S.T., J.B. Mitton, and T.D. Paine, *Strong differentiation in mitochondrial DNA of Dendroctonus brevicomis (Coleoptera : Scolytidae) on different subspecies of ponderosa pine.* Annals of the Entomological Society of America, 1999. **92**(2): p. 193-197.

26. Conkle, M.T. and W.B. Critchfield, *Genetic variation and hybridization of ponderosa pine*, in *Ponderosa pine: the species and its management*, D.M. Baumgartner and J.E. Lotan, Editors. 1988, Washington State University Press: Pullman, Washington. p. 27-43.

27. Betancourt, J.L., T.R. Van Devender, and P.S. Martin, *Packrat middens: the last 40,000 years of biotic change.* 1990, Tucson, AZ: University of Arizona Press.

28. Latta, R.G. and J.B. Mitton, *Historical separation and present gene flow through a zone of secondary contact in ponderosa pine.* Evolution, 1999. **53**(3): p. 769-776.

29. Potter, K., et al., *Nuclear genetic variation across the range of ponderosa pine (Pinus ponderosa): Phylogeographic, taxonomic and conservation implications.* Tree Genetics & Genomes, 2015. **11**(3): p. 1-23.

30. de Fine Licht, H.H., J.J. Boomsma, and D.K. Aanen, *Asymmetric interaction specificity between two sympatric termites and their fungal symbionts.* Ecological Entomology, 2007. **32**(1): p. 76-81.

31. Wood, S.L., *The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae).* Great Basin Naturalist, 1982. **6**: p. 1-1359.

32. Bracewell, R.R., et al., *Cryptic postzygotic isolation in an eruptive species of bark beetle (Dendroctonus ponderosae).* Evolution, 2011. **65**(4): p. 961-75.

33. Bentz, B.J., J.A. Logan, and J.C. Vandygriff, *Latitudinal variation in Dendroctonus ponderosae (Coleoptera : Scolytidae) development time and adult size.* Canadian Entomologist, 2001. **133**(3): p. 375-387.

34. Honek, A., *Intraspecific Variation in Body Size and Fecundity in Insects - a General Relationship.* Oikos, 1993. **66**(3): p. 483-492.

35. White, T.J., et al., *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*, in *PCR Protocols: A Guide to Methods and Applications*, M.D. Innis, et al., Editors. 1990, Academic Press, Inc.: New York. p. 315-322.

36. Vilgalys, R. and M. Hester, *Rapid Genetic Identification and Mapping of Enzymatically Amplified Ribosomal DNA from Several Cryptococcus Species.* Journal of Bacteriology, 1990. **172**(8): p. 4238-4246.

37. Anderbrant, O., *Gallery Construction and Oviposition of the Bark Beetle Ips-Typographus (Coleoptera, Scolytidae) at Different Breeding Densities.* Ecological Entomology, 1990. **15**(1): p. 1-8.

38. Amman, G.D., *Mountain Pine Beetle Coleoptera-Scolytidae Brood Production in Relation to Thickness of Lodgepole Pine Phloem.* Journal of Economic Entomology, 1972. **65**(1): p. 138-&.
39. Pinheiro, J., et al., *Package 'nlme'*. 2015.
40. Foelker, C.J. and R.W. Hofstetter, *Heritability, Fecundity, and Sexual Size Dimorphism in Four Species of Bark Beetles (Coleoptera: Curculionidae: Scolytinae).* Annals of the Entomological Society of America, 2014. **107**(1): p. 143-151.
41. Hothorn, T., et al., *multcomp: Simultaneous Inference in General Parametric Models, Version 1.3-1.* 2014.
42. Mueller, U.G., *Symbiont recruitment versus ant-symbiont co-evolution in the attine ant-microbe symbiosis.* Current Opinion in Microbiology, 2012. **15**(3): p. 269-277.
43. Barras, S.J., *Reduction of Progeny and Development in Southern Pine Beetle Following Removal of Symbiotic Fungi.* Canadian Entomologist, 1973. **105**(10): p. 1295-1299.
44. Goldhammer, D.S., F.M. Stephen, and T.D. Paine, *The Effect of the Fungi Ceratocystis Minor (Hedgecock) Hunt, Ceratocystis Minor (Hedgecock) Hunt Var Barrasii Taylor, and Sjb-122 on Reproduction of the Southern Pine-Beetle, Dendroctonus Frontalis Zimmermann (Coleoptera, Scolytidae).* Canadian Entomologist, 1990. **122**(5-6): p. 407-418.
45. Kaneko, T. and K. Takagi, *Biology of some scolytid ambrosia beetles attacking tea plants.* Applied Entomology and Zoology, 1966. **4**(1): p. 173-176.
46. Dysthe, J.C., R.R. Bracewell, and D.L. Six, *Temperature effects on growth of fungal symbionts of the western pine beetle, Dendroctonus brevicomis.* Fungal Ecology, 2015. **17**: p. 62-68.

47. Six, D.L. and K.D. Klepzig, *Dendroctonus bark beetles as model systems for studies on symbiosis.* Symbiosis, 2004. **37**(1-3): p. 207-232.

48. Six, D.L. and T.D. Paine, *Ophiostoma clavigerum is the mycangial fungus of the Jeffrey pine beetle, Dendroctonus jeffreyi.* Mycologia, 1997. **89**(6): p. 858-866.

49. Roe, A.D., et al., *Comparative phylogeography, genetic differentiation and contrasting reproductive modes in three fungal symbionts of a multipartite bark beetle symbiosis.* Molecular Ecology, 2011. **20**(3): p. 584-600.

50. Yuceer, C., et al., *Ultrastructure of the mycangium of the southern pine beetle, Dendroctonus frontalis (Coleoptera: Curculionidae, Scolytinae): complex morphology for complex interactions.* Acta Zoologica, 2011. **92**(3): p. 216-224.

51. Bleiker, K.P., et al., *Transport of fungal symbionts by mountain pine beetles.* Canadian Entomologist, 2009. **141**(5): p. 503-514.

52. Happ, G.M., C.M. Happ, and S.J. Barras, *Fine Structure of Prothoracic Mycangium, a Chamber for Culture of Symbiotic Fungi, in Southern Pine Beetle, Dendroctonus-Frontalis.* Tissue & Cell, 1971. **3**(2): p. 295-&.

Table 1. Mean (SE) of parent and larval gallery lengths, total number of offspring produced and proportion of female to male offspring produced by western pine beetle developing with no fungi or fungal treatments. Values in the same column followed by the same letter are not significantly different (Tukey's HSD test, α = 0.05)

Treatment	N	Parent gallery length (cm)	Larval tunneling (cm)	Total offspring	Proportion female
Natal	14	65.96 (5.94)a	1.53 (0.31)a	23.79 (6.93)a	0.437(0.19)a
Non-natal	9	85.86 (13.10)a	1.46 (0.33)a	22.89 (6.56)a	0.521(0.19)a
No fungus	12	62.86 (6.77)a	2.05 (0.44)a	0.25 (0.17)b	*

* Unable to estimate due to too few individuals.

Table 2. Results from GLM analysis of the influence of fungal treatment (natal, non-natal, no fungus) on four measures of western pine beetle reproductive success (Response variable).

Response variable	Factor	Wald Chi Square	*df*	*p-value*
Parent gallery length	Intercept	85.5	1	<0.0001
	Treatment	4	2	0.14
Larval tunneling	Intercept	20.8	1	<0.0001
	Treatment	1.5	2	0.47
Total offspring	Intercept	3344.4	1	<0.0001
	Treatment	61.7	2	<0.0001
Proportion female	Intercept	3.4	1	0.066
	Treatment	3.2	1	0.074

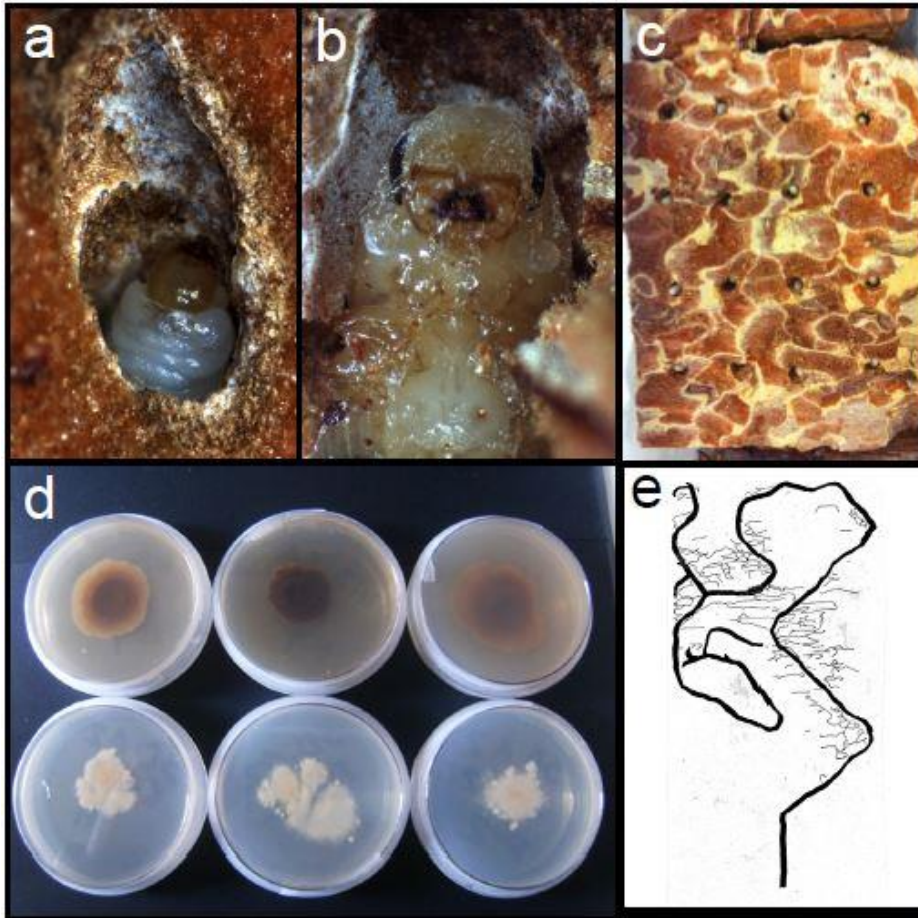

Figure 1. a) Western pine beetle feed heavily on symbiotic fungi while developing in ponderosa pine bark (fungi seen as white mats in larval tunnel). b) During pupation in the bark, fungal spores line the pupal chamber for incorporation into the mycangia after metamorphosis. c) For our experiments, we created pseudo-pupal chambers in ponderosa pine bark to rear aposymbiotic adult western pine beetles. Each chamber contains a pupa. d) Two genetically, geographically, and phenotypically distinct isolates of *Entomocorticium* sp. B (haplotype A = natal, shown above, haplotype C = non-natal, shown below), were used to test for adaptation of beetles to particular fungi. e) Example of transparency tracings of typical parent tunnels (thick lines) and larval tunnels (thin lines) of a western pine beetle gallery from one of the fungus treatments.

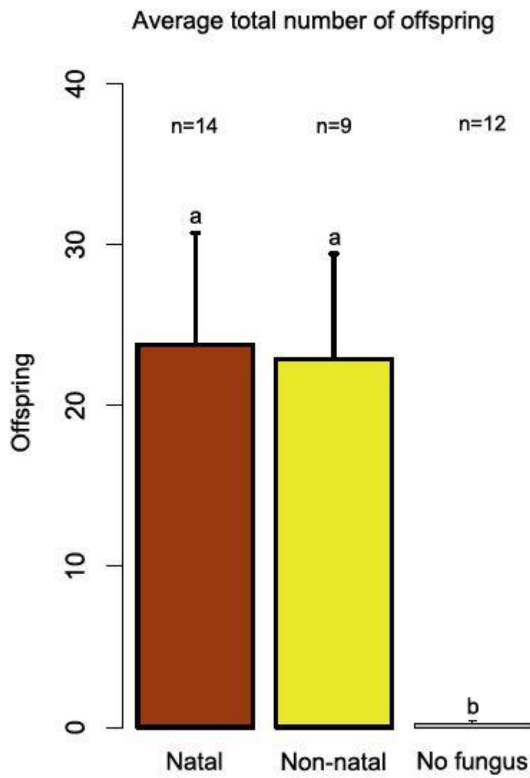

Figure 2. Average total number of offspring (error bars = SEM) from the natal, non-natal, and no fungus treatments. Number of male/female pairs per treatment are denoted above their respective bar. Bars with the same letter are not statistically significantly different from one another (Tukey HSD test).

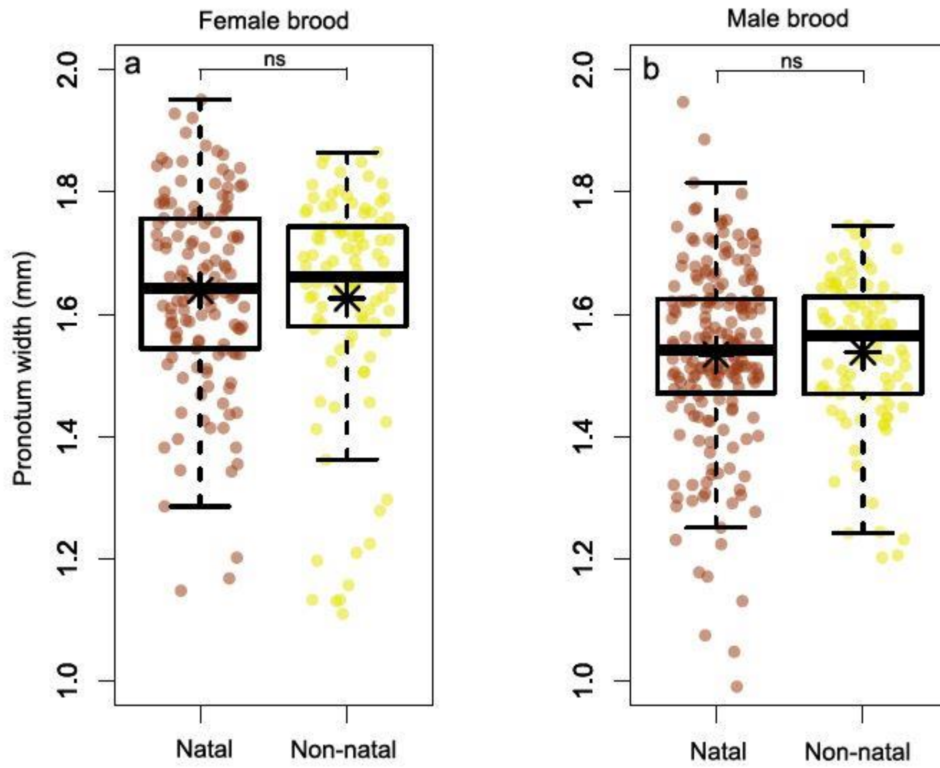

Figure 3. Comparison of size (pronotum width) of brood a) females and b) males from the natal and non-natal fungal treatments. Each pronotum measure is represented by a point on their respective boxplot and the mean size per treatment is denoted with an asterisk. Not significant = ns.

Appendix 1.

Treatment	Replicate	Total brood	Total females	Isolated	Positive for natal fungus*	Isolated females positive for natal fungus (%)
natal	3	55	26	8	4	50%
natal	5	37	14	6	5	83%
natal	6	28	12	6	4	67%
natal	8	18	12	4	3	75%
natal	10	48	18	10	8	80%
natal	11	6	1	1	1	100%
natal	12	44	16	4	0	0%
natal	13	8	4	3	2	67%
natal	14	77	36	4	4	100%
natal	15	1	1	1	0	0%
natal	16	3	2	2	0	0%
non-natal	2	10	4	4	0	0%
non-natal	3	8	4	4	3	75%
non-natal	6	22	3	3	0	0%
non-natal	7	31	20	14	0	0%
non-natal	8	30	15	14	0	0%
non-natal	10	25	13	10	0	0%
non-natal	13	15	12	11	0	0%
non-natal	15	112	20	6	4	67%
non-natal	16	66	40	9	0	0%
non-natal	17	51	28	6	4	67%
no fungus	2	1	1	0	0	NA
no fungus	7	2	2	1	0	0%
no fungus	16	29	11	5	4	80%

*Identified by sequencing ITS2-LSU region of representative isolate and morphotyping remaining isolates

CHAPTER 3

Cascading speciation among mutualists and antagonists in a tree-beetle-fungal interaction

ABSTRACT

Cascading cospeciation occurs when interacting species diverge in parallel as a result of divergence in one species promoting adaptive differentiation in other interacting species. This cascade can ripple down through multiple species and cause divergence across multiple trophic levels. Here, we investigated the western pine beetle system to determine if there was evidence of genetic structure and cascading cospeciation occurring between the beetle (*Dendroctonus brevicomis*), its primary host tree (*Pinus ponderosa*), and the beetle's fungal mutualists (*Ceratocystiopsis brevicomi* and *Entomocorticium* sp. B). We sequenced and assembled draft genomes for the beetle and fungi and then generated reduced representation genomic data (RADseq) from range-wide samples of these species. Combined with previously published data for the host tree, we found clear evidence that the tree, the beetle, and the fungal symbionts were all genetically structured into at least two distinct groups that correspond with previously described sub-species boundaries of the tree. Further, we identified three highly distinct species of *Entomocorticium* sp. B; two that geographically co-occur. Our results highlight how periods of geographic isolation of coevolving species can facilitate the evolution of genetic differentiation and further, that coevolution in isolation likely enhances the evolution of reproductive isolation among diverging lineages of beetle and fungi. Our study also provides important insights into the complex western pine beetle-fungal symbiosis.

INTRODUCTION

A central goal of evolutionary biology is to understand the processes that lead to the diversity of life. Speciation, the splitting of one lineage into two, is the fundamental process that creates diversity. While our understanding of speciation has increased dramatically over the last few decades [1], most genetic research on speciation has focused on understanding the evolution of reproductive isolation between pairs of diverging taxa. Far fewer investigations have looked at how interacting complexes of species may promote or constrain processes that lead to diversification [2]. Recently, the idea that increases in species diversity itself may promote speciation by creating new ecological niches has gained considerable attention [3, 4]. So called examples of 'sequential' or 'cascading' divergence and speciation have been described in systems where an herbivorous insect shifts to a new host plant, diverges, and becomes reproductively isolated from its progenitor. In sequential fashion, parasitoids specialized on the herbivore also diverge and become reproductively isolated [5, 6]. The end result of this process is codifferentiation and ultimately cospeciation across multiple trophic levels.

Cascading cospeciation among multiple interacting species (or 'phylogenetic cascades' [2]) may extended beyond plant-herbivore-parasitoid systems, however, this possibility remains largely unexplored. One type of ubiquitous multi-trophic interaction where such cascades may be common are plant-herbivore-microbe systems. Microbes commonly form associations with other organisms, including insects. For many insects, these associations play a critical role in their adaptation to specific ecological niches [7, 8]. Further, there are now examples where particular endosymbiotic bacterial genotypes aid in insect adaptation to host plants [9]. For plant-feeding insects involved in endosymbiotic relationships with bacteria, it is easy to envision how a host plant shift could result in divergence of the insect and subsequent isolation of the endosymbiont.

Both theoretical and empirical studies have demonstrated the intimate link between insect and endosymbiont cospeciation [10, 11] and cospeciation has even been shown to extend across multiple endosymbiotic taxa [12]. However, many insect-microbe symbioses are ectosymbioses that occur with bacteria or fungi [7]. In ectosymbiosis, even those with vertical symbiont transmission, the association is often more loose and symbionts can be lost or swapped out over evolutionary time. Because ectosymbioses may be more fluid, the extent to which coevolution (reciprocal adaptation) results in stable long-term associations that drive cospeciation remains unknown. One ecologically important and well-known example of an insect-microbe ectosymbiosis involves the so called 'fungus farming' insects that cultivate fungi for food [7, 13]. These insect-fungal interactions can be highly stable and result in cospeciation over broad evolutionary timescales [14], although symbiont turnover also occurs in these systems [15].

The ponderosa pine-western pine beetle-fungal symbiosis provides a unique opportunity to understand coevolution, cospeciation and cascading differentiation among multiple interacting species. The host tree, ponderosa pine (*Pinus ponderosa*), is broadly distributed across the western United States (Fig. 1A). Within this species there exists two genetically and phenotypically distinct varieties that have historically been considered different subspecies [16] (Fig. 2A). Recent population genetic analyses support the existence of two genetically distinct lineages [17] that formed during the Pleistocene [18]. Palynological [19] and genetic evidence [17, 20] suggests the subspecies formed in allopatry in two isolated glacial refugia in the southern portions of their current distributions [21]. As climate warmed, the two subspecies expanded their distributions northward [18, 19]. There are a number of phenotypic differences within and between these taxa (summarized in [17]), including variation in their defensive chemicals, particularly their xylem resin monoterpenes [22].

The western pine beetle is one of the most significant pests of ponderosa pine [23] (Fig. 1B). Except in a very small portion of the beetle's range where it reproduces in the closely related *Pinus coulteri*, the western pine beetle only attacks and reproduces in ponderosa pine [24]. Therefore, any host-associated adaptation and differentiation in the western pine beetle is likely to be driven by interactions with this tree. The ability of the beetle to recognize and colonize host trees is intimately linked to host tree chemistry [25]. Western pine beetles stage pheromone-mediated attacks of living host trees using hundreds to thousands of individuals to overcome the tree's physical and chemical defenses. After exhausting these defenses, the beetle reproduces within the tree, first feeding in the phloem and then later moving into the outer bark (Fig. 1B). Previous studies found substantial levels of mitochondrial DNA (mtDNA) sequence divergence (~7-9%) between beetles associated with the two ponderosa pine lineages, suggesting parallel plant-herbivore divergence in this system [26, 27].

The beetle is involved in an obligate mutualism with two species of symbiotic fungi [28-31]. The beetle benefits by supplementing its nutrient poor diet through feeding on fungi inoculated into the tree during the initial stages of attack while the fungi benefit by gaining transportation to host trees (Fig 1C). This symbiosis is maintained primarily via vertical transmission of the symbiont from mother to offspring via specialize structures in the adult integument called mycangia. One species, *Ceratocystiopsis brevicomi*, is from an order of ascomycetes (Ophiostomatales) associated with bark beetles [28]. The other species, *Entomocorticium* sp. B, is in a group of mostly undescribed and poorly known basidiomycetes that all appear to be facultative or obligate symbionts of bark beetles [29]. Both species of fungi (hereafter the ascomycete or basidiomycete) appear morphologically adapted to beetle feeding and dispersal by beetles [32] and neither is known to exist outside the western pine beetle

system. However, they are not functionally equivalent symbionts. The basidiomycete appears to be the more frequent and beneficial partner [33]. Further, the basidiomycete is capable of extracting nutrients from the outer tree bark [34] where beetle larva develop; a substrate that neither the insect nor the ascomycete can metabolize. In total, the ponderosa pine-western pine beetle-fungal symbiosis is best described as a network of antagonistic and mutualistic species interactions spanning three taxonomic kingdoms (Fig. 1D). Here we combine whole genome assemblies and genome-wide reduced representation sequencing (RADseq) from samples spanning the range of the beetle, basidiomycete, and ascomycete with previously published population genetic data for the tree and test for cascading speciation across this species interaction network.

RESULTS

Beetle, basidiomycete and ascomycete genome assemblies

The close beetle-fungal symbiosis makes it difficult to fully isolate the symbionts from the beetle. This issue is particularly worrisome for the analysis of RADseq markers based on *de novo* assemblies where contamination from one or more of the interacting species could confound our analyses of codifferentiation. To overcome this issue we first generated whole genome assembles for the western pine beetle, the basidiomycete and the ascomycete. We sequenced the beetle to a mean coverage of 151× and assembled the genome with a combination of overlapping Illumina paired-end read and mate-pair libraries using the ALLPATHS-LG assembler [35]. Our assembled genome had a scaffold N50 of 5 kb, and consisted of 35,469 scaffolds, with the maximum scaffold size of 540,064 bp. The total length of the assembly was 130 Mb (Table 1). Although highly fragmented, we were able to identify 242 of the 248 (98%) core eukaryotic

genes in the assembly, with 212 (85%) being complete [36]. For comparison, 96% of the core eukaryotic genes were recovered in assembly of the closely related mountain pine beetle (*Dendroctonus ponderosae*) [37]. We estimate the western pine beetle genome to be 192 Mb (Table 1), which is similar to the mountain pine beetle (204 Mb) [37].

We sequenced the basidiomycete genome to 81× coverage. Using the same library types and assembler, we obtained a substantially better assembly for the basidiomycete with a scaffold N50 of 54 kb, a total of 1,248 scaffolds, and a max scaffold size of 302,230 bp (Table 1). The total length of the assembly was 36 Mb. This size is only slightly smaller than the two closest free-living fungal relatives that have been sequenced and assembled using the same assembly algorithm (two *Peniophora* spp. with estimated genome sizes of 46 Mb and 48 Mb, (http://genome.jgi.doe.gov)). Interestingly, the estimated genome size of the basidiomycete was nearly double that of the final assembly length (Table 1), suggesting the basidiomycete genome contains many repetitive elements that complicate the assembly. Despite this, we were able to identify 245 of 248 (99%) core eukaryotic genes in the basiciomycete assembly, with 240 being complete (97%) [36].

We then sequenced the ascomycete genome to 148× coverage using methods outlined above, and obtained a good assembly with a scaffold N50 of 1,358 kb, a total of 198 scaffolds, and a max scaffold size of 3,492,413 bp (Table 1). The total length of the assembly was 21 Mb (Table 1). The estimated genome size was 29 Mb which is similar to other close relatives (three Ophiostomatoid fungi with estimated genome sizes of 27, 30 and 33 Mb, (http://genome.jgi.doe.gov)). We were able to identify 241 of 248 (97%) core eukaryotic genes in the ascomycete assembly, with 235 being complete (95%) [36].

Codivergence of the tree, beetle and fungal symbionts

To characterize the genetic structure of the four interacting species, we obtained range-wide genetic data for the tree, beetle, and the two fungal symbionts. For the tree, we obtained microsatellite data from a previously published geographically comprehensive analysis (n = 428 trees [17]). We generated genome-wide reduced representation RADseq data for the beetle (n = 156), three closely related beetle species (*D. approximatus*, *D. adjunctus*, and *D. frontalis*), the ascomycete symbiont (n = 19) and an outgroup (*C. ranaculosus*), and the basidiomycete symbiont (n = 36, including a reference isolate of *E.* sp. B (B1037)) and four outgroups (*E.* sp. A, C, G, and H); Supplemental Table 1, Fig. 2A). We sequenced the beetles to a mean coverage of 13× (min = 4.4×, max = 40.1×), the ascomycete isolates to a mean coverage of 53× (min = 28.6×, max = 76.7×) and the basidiomycete isolates to a mean coverage of 39× (min = 10.3×, max = 78.5×). We then mapped the RADseq data to the *de novo* assembled reference genomes for each species. On average, 87%, 95%, and 80% of cleaned RADseq reads mapped to the basidiomycete, ascomycete, and beetle genome assemblies, respectively. This fairly high mapping rate indicated that our genome assemblies and RADseq data were both of high quality. After filtering and excluding outgroups, we identified 65,624, 6,874 and 6,424 SNVs (single nucleotide variants), in the ascomycete, basidiomycete, and beetle, respectively.

To test for concordant genetic structure among the four species we first performed STRUCTURE [38] and ADMIXTURE [39] analyses independently for the tree, beetle, and the two fungal mutualists. In three of the four taxa (tree, beetle and ascomycete), the optimal number of clusters (*K*) was unambiguously two (Fig. 2B) and split individuals into groups consistent with previously described host tree subspecies boundaries (hereafter referred to as East and West groups; Fig. 2A). For the basidiomycete, we found support for three groups with clear clustering

of individuals into East and West groups and additional partitioning of West individuals. The West basidiomycetes were unambiguously assigned into two broadly geographically overlapping groups which were from various collection locations (CQ, KE, SU, SierraIII, LA, MI, Sierra, SB).

Next we used principal component analyses (PCA) to further visualize fine-scale genetic structure. Consistent with STRUCTURE and ADMIXTURE results, PCA clearly resolved East and West groups for the tree, beetle, ascomycete and basidiomycete (Fig. 3). The second principal component for the West tree (Fig. 3A; green ellipse), beetle (Fig. 3B; green ellipse), and ascomycete (Fig. 3C, green ellipse) showed some evidence of a south-to-north cline in genetic variation with the most southern population (SB) showing strong differentiation (Fig. 3). For the basidiomycete (Fig. 3D), the second principal component split into two groups the two clusters of individuals identified in previous STRUCTURE analyses.

To directly test for codivergence between the tree, beetle, and ascomycete (here excluding the basidiomycete, see below), we tested for correlations among the three genetic distance matrices using a framework [40] that has been used to detect correlated genetic structure in other bark beetle-fungus symbioses [41, 42]. This analysis was restricted to nine sites (six West and three East) where we had genetic data for the tree, beetle, and ascomycete. Using this approach, we strongly rejected the global null hypothesis of incongruence among the distance matrices ($W = 0.82$, Friedman's $\chi^2 = 86.44$, $p = 0.001$). *A posteriori* permutation tests of the influence of individual distance matrices on overall concordance indicated the strongest correlation was between beetle and ascomycete ($r = 0.78$, $p = 0.001$), followed by beetle and tree ($r = 0.76$, $p = 0.010$), and then tree and ascomycete ($r = 0.66$, $p = 0.007$).

Our population genomic analyses revealed geographic structuring within species and strongly correlated East-West genetic differentiation for all species with the strongest associations occurring between the beetle and its fungal ascomycete symbiont. Next we estimated rooted maximum likelihood trees for the beetle and the two symbiotic fungi to better resolve the evolutionary history of codivergence (Fig. 4). The East and West populations partitioned into reciprocally monophyletic groups defining the deepest phylogenetic split within both the beetle and the basidiomycete. Finer scale geographic structuring within East and West was largely absent within the beetle, except for monophyly of two geographically isolated populations (SB and MTC) from the southern portion of the East and West groups. Consistent with our population genetic analyses there were three phylogenetically distinct basidiomycete lineages (previously identified as haplotype A, B and C [33], and hereafter designated as B_1, B_2, and B_3, respectively) associated with the beetle with very strong support for more recent ancestry between the two overlapping and broadly distributed West lineages (Fig. 4). The pattern of reciprocal East-West monophyly was not found for the ascomycete, revealing an important and strongly supported phylogeographic relationship. East ascomycetes are nested within the West group (i.e, paraphyletic) and more closely related to the most southern West population (SB), thereby providing evidence that East ascomycete individuals are derived from West.

Given the presence of genetically differentiated East and West groups in all four species, we then characterized the levels of genetic diversity, differentiation, and level of DNA sequence divergence among these groups. Of particular interest were the three distinct basidiomycete lineages we had identified in previous analyses. The basidiomycete is thought to be the more beneficial symbiont and is the most common fungal associate [33]. The presence of more than one West lineage with no clear geographic association was a surprise. Further, both lineages

were found to co-occur at sites. At SB and CQ, beetles were collected that were found to harbor one or the other lineage (Fig. 3D). The broad co-occurrence of two West basidiomycete lineages was found previously, although at the time, only the ITS region had been sequenced and there was few differences between the lineages [33]. To characterize genetic differentiation between the three basidiomycete lineages, we calculated Weir and Cockerham's F_{st} [43] and found universally high levels of differentiation. We found that F_{st} between the two West lineages (B_1 and B_2) was = 0.92. Comparisons of East and West lineages were even higher with F_{st} between B_1 and B_3 = 0.96 and between B_2 and B_3 = 0.97. Although the basidiomycete had many fixed differences in the three lineages, there were exceptionally low levels of nucleotide diversity (π) in all lineages (Table 2). In combination with high levels of differentiation and low levels of diversity, the three lineages showed fairly low levels of overall sequence divergence. D_{xy} between the two West lineages (B_1 and B_2) was = 0.17%. D_{xy} between East and West lineages was higher, and between B_1 and B_3 was = 0.33%, and between B_2 and B_3 was = 0.32%. Our results suggest that the western pine beetle harbors three highly differentiated lineages of the basidiomycete which show low levels of divergence from one another and all have exceptionally low levels of diversity.

In comparisons of East and West ascomycetes, we found that differentiation was moderate and F_{st} was = 0.33. We found that absolute sequence divergence (D_{xy}) was comparable to the basidiomycete, yet was slightly higher at 0.43%. Further, we found that nucleotide diversity for ascomycete was substantially higher in West than in East. For West, the ascomycete diversity was found to be 18× and 29× higher than the co-occuring basidiomycetes B_1 and B_2, respectively (Table 2). Further, the Tajima's D estimate for West ascomycetes was fairly negative (Table 2) which would be consistent with a recent population expansion. For the beetle,

we found absolute divergence (D_{xy}) to be substantially higher between East and West beetles (1.37%). F_{st} was also fairly high at 0.51. Interestingly, we also found evidence of reduced diversity in East compared to West beetles (Table 2). This pattern mirrored the diversity patterns seen in the ascomycete. The lower nucleotide diversity in East for both the beetle and the ascomycete suggest that similar population genetic forces (e.g., a bottleneck or founder event) may have historically reduced genetic diversity in both species.

Fungal symbiont recombination

For fungal mutualists that are primarily vertically transmitted from parent to offspring, it has often been assumed reproduction is asexual [7]. The advantage of asexual reproduction in the short term is clear; if an advantageous symbiont genotype arises, recombination may break up the combination of alleles that produced the good symbiont. However, the long term repercussions of asexuality could be severe since the symbiont would be unable to purge deleterious mutations (Muller's ratchet). Recent evidence has challenged the idea that vertically transmitted fungal symbionts are exclusively asexual [44] and indeed, recombination has been found to occur in the few bark beetle symbionts thus far investigated [42, 45].

For the ascomycete, we found clear evidence of recombination (Pairwise Homoplasy Index (PHI), $p < 0.005$, and four gamete test). This result was not altogether surprising given our own observations of sexual structures in this fungus and previous work describing sexual structures in culture [28]. For the basidiomycete, we also found evidence of recombination occurring within the three distinct lineages (PHI test $p < 0.005$, and four gamete test); however, there was no evidence of recombination occurring between the lineages. Taken together with the low genetic diversity observed, our results suggest the basidiomycete is highly selfing or

primarily asexual, with infrequent bouts of recombination via either sexual or parasexual means. Although sexual structures (basidia) have not been identified in the basidiomycete, a closely related species does produce basidia in culture [46]. Infrequent bouts of recombination may be advantageous for a fungus in a tightly linked symbiosis. The high level of selfing or asexuality may help maintain favorable genotypic combinations that promote stability and could be selected for by the beetle. However, rare recombination events may help alleviate issues associated with obligate selfing or asexuality and the accumulation of deleterious mutations [47].

The lack of recombination between basidiomycete B_1 and basidiomycete B_2, the two less divergent and broadly co-occurring lineages, argues that all three basidiomycete lineages are completely reproductively isolated from one another. From a speciation standpoint, the presence of two co-occurring, reproductively isolated symbiotic lineages is interesting given that the basidiomycete is not thought to occur outside of the symbiosis. Therefore, this speciation event would have had to have taken place during its association with West beetles. West beetles were found to have little population genetic structure (with the exception of SB) suggesting this speciation event may have even occurred in sympatry.

DISCUSSION

The population genetic analyses and phylogenetic relationships for the host tree, its antagonistic beetle, and the fungal mutualists all identified East and West groups. Our results clearly found genetically distinct groups of the tree which corresponded to previously described subspecies [16], and confirmed the results from Potter et al. [17]. Further, our results provided conclusive evidence from the nuclear genome that the beetle is indeed highly differentiated into two groups that are isolated on the two tree subspecies [26]. Corresponding divergence among the fungal

mutualists provides an additional layer of codifferentiation in the system. Both fungal symbionts were found structured into East and West groups and we identified an additional basidiomycete lineage in the West.

A process whereby divergence in one species results in a cascade of divergence among multiple closely associated organisms is intuitive [2]; however, cases demonstrating this are rare and primarily restricted to plant-herbivore-parasitoid systems [5, 6]. The data presented above strongly suggest that East and West populations of the tree, beetle, and the fungal symbionts are coevolving and genetically isolated from one another. Whether the accumulated genetic differences translate to differences that would result in reproductive isolation if East and West groups were to co-occur is only partially known. For the tree, there is evidence of postzgotic isolation in the form of reduced seed-set when crossing the two subspecies [16] and patterns of reduced gene flow across a zone of secondary contact argues for some degree of reproductive isolation [18]. For the beetle, there is only one published account of crossing East and West beetles and there was evidence of asymmetric hybrid inviability [48]. However, small sample sizes and a failure to characterize the full range of postzygotic isolation (intrinsic/extrinsic, inviability/sterility) makes the strength of isolation unclear. For the two species of fungi, compatibility tests have not been conducted, and may be impossible give the difficulties of getting fungal symbionts to produce fruiting bodies outside the symbiosis and in artificial media. Regardless, the genetic data argues strongly that there is complete reproductive isolation in at least the basidiomycete.

An important distinction between cospeciation (which has been demonstrated repeatedly in endosymbiotic systems) and the special case of cascading cospeciation is that the former can be due to purely ‘passive’ process of genetic differentiation (i.e., mutation and drift) of co-

isolated species while the latter is a direct result of interspecific adaptation driving codifferentiation [2, 6, 49]. One prediction of purely 'passive' cospeciation is that although reproductive isolation may evolve between diverging lineages, the underlying interspecific interactions would not necessarily evolve or result in further reproductive isolation. Therefore, evidence of coevolution that drives divergence and further reproductive isolation in the system, even if occurring in isolation, supports that coevolving differences in the underling interspecific interactions are facilitating divergence and the speciation event. We have previously shown that West beetles are incapable of incorporating the East lineage of the basidiomycete into their mycangia suggesting that beetles have evolved specificity to particular fungal genotypes [30]. Even if East and West beetles were to come into contact and produce viable hybrids, it is unclear if hybrid beetles could maintain their association with any lineage of basidiomycete; a fungal symbiont crucial for successful development [30]. Further, East and West trees have evolved qualitative and quantitative differences in tree defensive chemistry [22]. For the beetle, host tree chemical profiles are important for host tree recognition and during tree colonization, defensive chemicals are converted to pheromones [50]. Tree chemicals that play a role in host tree colonization in the West [50], have been found to differ in quality and quantity in East trees [22]. Therefore, if beetles were to encounter trees in which they do not share an evolutionary history, differences in tree chemistry may lead to difficulties finding suitable hosts and in attracting conspecifics. Evidence of an evolved difference in beetle response to tree chemistry has even been partially demonstrated. Attractant lures developed to monitor East beetles were found to be far more effective in West when fine-tuned to match West tree chemistry [51]. In total, the nature of the tightly linked tree-beetle-fungal system, with some evidence for coevolution occurring between tree-beetle-fungi suggests this system is best described as a cascade of codifferentiation.

How often cospeciation occurs between trees, beetles, and fungal symbionts, appears to depend on the groups in question. For the beetles (genus *Dendroctonus*) and their coniferous host trees (*Pinus*, *Larix* and *Picea*), there exists some phylogenetic conservatism in host tree species use and closely related beetle species tend to occur on closely related tree species [27]. However, there is no evidence of widespread cospeciation among trees and beetles [27]. For the beetles and fungal symbionts, and particularly beetles in tight associations with fungal symbionts (mycangia-bearing *Dendroctonus*), patterns of cospeciation with ascomycetes are indeed present [52, 53]. However, there is no evidence of strict beetle-fungal cospeciation and there is some evidence that beetles occasionally swap or acquire additional symbionts through time [53]. For the other group of fungal symbionts, the basidiomycetes, we currently have a very poor understanding of evolutionary relationships [29] and no formal cophylogenetic analyses have been performed. A more widespread and thorough analysis of all beetle (*Dendroctonus*) mycangial fungi is desperately needed to tease apart the full breadth of fungal associations and patterns of cospeciation. Regardless of the overarching tree-beetle-fungal symbiont cospeciation patterns over longer evolutionary time frames, the ponderosa pine-western pine beetle-fungal symbiosis provides an important glimpse of codifferentiation during speciation and emphasizes how geographic isolation of highly specialized organisms can promote diversification of multiple tightly-linked species.

METHODS

Beetle/fungal collections.

Western pine beetles and their fungal symbionts were collected in the summer of 2011 (Supplemental Table 1). Live beetles were collected using a variety of methods and sampling at

all locations was done in a manner to avoid collecting related beetles (Supplemental Table 1). Live beetles were transported to the University of Montana, where we isolated the fungi from the mycangia of beetles prior to preserving them in 95% EtOH and placing them -80 ºC. Mycangial fungi were isolated from beetles using methods described previously [33]. To generate a pure fungal culture of a single individual, we isolated a single spore for each of the *Ceratocystiopsis* samples and then generated cultures from the initial spore. For *Entomocorticium* samples, we used the hyphal-tip isolation technique since these fungi do not sporulate in culture. A single hyphal tip could potentially harbor one haploid nucleus (monkaryon), two different haploid nuclei (dikaryon), or one diploid nucleus. Downstream analyses were used to distinguish the state of *Entomocorticium* isolates (below). Hyphal tips, or single spores, were cultured on 2% MEA for a few weeks, and then an actively growing portion of the mycelium was transferred to liquid media (2% malt extract and sterile water) and grown for an additional month so that ample quantities of fungal tissue were available for DNA extractions. Bark beetle outgroups (*D. approximatus*, *D. frontalis*, and *D. adjunctus*) were all collected during western pine beetle collections in 2011 and placed immediately in 95% EtOH. *Dendroctonus approximatus* and *D. adjunctus* were collected near Beaver, UT (38° 22' N, 112° 31' W) while *D. frontalis* was collected near Morenci, AZ (33° 28' N, 109° 22' W). All fungal outgroup collections are detailed in Bracewell et al. [33].

DNA extractions

DNA was extracted from adult beetles using an Omega E.Z.N.A tissue DNA kit (Prod. No. D3396-01, Omega Bio-Tek, Norcross, GA, USA) and manufacturers recommended protocols. Due to the small size of the beetle and the need for large quantities of DNA for the

RADseq protocol and whole genome sequencing, we extracted DNA from as much tissue as possible while attempting to avoid non-target DNA. Female beetle tissue used in DNA extractions included the head, the posterior portion of the pronotum (therefore excluding the mycangia) and the anterior portion of the thorax. Male beetles lack a mycangium and therefore DNA was extracted from the head to the anterior portion of the thorax (excluding elytra and gut). For all fungal samples, DNA was extracted by taking fungal tissue from liquid media and lightly drying the tissue at ambient temperatures to remove excess water. Approximately 100 mg of slightly moist fungal tissue was then bead-beat with ceramic balls to help break apart the cells (Geno/Grinder 2000, Spex Certiprep, Metuchen, NJ). DNA was then extracted from the bead-beat tissue using the Qiagen DNeasy Plant Mini Kit (Cat. No. 69104) and manufacturers recommended protocols.

Western pine beetle whole genome library preparation and sequencing

To use the ALLPATHS-LG [35] assembler to generate a draft of the western pine beetle genome we generated both an overlapping read library (Illumina 100bp PE insert size of ~180 bp), and a mate pair library (Illumina 100bp PE insert size of ~3kb). We chose one individual male western pine beetle from the OL population (Table 1) to construct both libraries. Given the small quantities of DNA extracted from one beetle, and the large quantity needed to construct mate-pair libraries, we whole genome-amplified the sample. Whole genome amplifications were performed using the Illustra GenomiPhi V2 DNA Amplificiation Kit (product number 25-6600-31, GE Healthcare Life Sciences, Pittsburgh, Pennsylvania, USA) and manufacturers recommended protocols. Whole genome amplified DNA was used to construct a mate pair library which was prepared by GENEWIZ Inc. (South Plainfield, New Jersey, USA) using the

Illumina Nextera Mate Pair kit (catalog number FC-132-1001) and recommended manufacturers protocols. For the overlapping read library, the original unamplified DNA extraction was initially sent for library preparation and sequencing. Unfortunately, during transport or storage, the sample degraded, so DNA from a different male beetle from the OL population was sent for library preparation and sequencing. The overlapping read library was prepared by the University of Utah Microarray and Genomic Analysis Core Facility (Salt Lake City, UT), using an Illumina TruSeq Nano DNA Sample Prep kit (catalog number FC-121-4001) and manufacturers recommended protocols. The sample was sequenced at the same facility using an Illumina HiSeq 2500 and 100bp PE reads. The mate pair library was sequenced at the University of Montana Genomics Core Facility (Missoula, MT), using 150bp PE reads on an Illumina MiSeq.

Entomocorticium *sp.* B *whole genome library preparation and sequencing*

To assemble a draft genome for *E* sp. B, we again generated both an overlapping read library and a mate pair library for an ALLPATHS-LG assembly. We used an isolate from the SierraII population (SierraII_2, CBS accession CBS 137838) that had previously been identified as haplotype A [33]. Approximately 1 µg of genomic DNA was prepared for Illumina sequencing using the NEXTflex DNA Sequencing Kit and DNA Barcodes by Bioo Scientific (Austin, TX) using methods outlined in Brown et al. [54]. To obtain overlapping reads, we size selected fragments using agarose gel excision to thereby ensure a large proportion of 100bp PE reads would produce overlapping reads. We amplified the size selected template library in 4 separate reactions to reduce to possibility of propagating any PCR error. PCR conditions were 98°C for 2m: 11X (98°C for 30s, 65°C for 30s, 72°C for 60s) with a final extension at 72°C for 4 minutes. The PCR products were combined, cleaned using AMPure XP beads (Beckman

Coulter, Brea CA) and sequenced using 100bp Paired end reads on an Illumina HiSeq 2000 at the Vincent J. Coates Genomics Sequencing Laboratory, Berkeley, CA. Mate-pair libraries were constructed using the Illumina Nextera Mate Pair kit (catalog number FC-132-1001) and size selected using a Pippin Prep system (Sage Science). The resulting ~4kb fragments were then circularized and sheared using a Covaris S220 sonicator (Covaris, Inc. MS, USA). The mate pair library was sequenced both as 101bp PE reads on an Illumina HiSeq 2000 at the University of Utah Microarray and Genomic Analysis Core Facility, Salt Lake City, UT, and 259bp PE reads on an Illumina MiSeq at the University of Montana Genomics Core Facility, (Missoula, MT).

Ceratocystiopsis brevicomi *whole genome library preparation and sequencing*

Library preparation, sequencing, and assembly of *C. brevicomi* was similar to the other fungus (above). We chose a *C. brevicomi* isolate from the SierraII population (SierraII_19, CBS accession CBS 137839) for genome assembly. Fewer PCR cycles (10) were needed for amplification of the *C. brevicomi* overlapping read library prior to sequencing.

Entomocorticium *sp.* B, Ceratocystiopsis brevicomi *and western pine beetle genome assemblies.*

For all species, all raw reads (both mate pair and overlapping) were first adapter trimmed and quality trimmed using default settings in SeqyClean version 1.8.10. An initial assembly was then performed with the cleaned and overlapping reads and the MaSuRCA genome assembler, version 3.1.0 [55]. We then used the Nextclip pipeline version 0.7 [56], along with our preliminary MaSuRCA-based draft, to process and map the mate-pair data and estimate the mean and standard deviation of insert size lengths for input into the final ALLPATHS-LG assembly. Mate pair reads found to be in the correct orientation based on Nextclip results were then used

along with our cleaned short reads to produce a final assembly with ALLPATHS-LG (release R48777).

Assembly statistics suggested our assembly for the *E.* sp. B and *C. brevicomi* were comparable to other published basidiomycete genomes (http://genome.jgi.doe.gov). However, our western pine beetle assembly was far more fragmented than the only other published *Dendroctonus* genome [37]. The western pine beetle karyotype is 2n = 5 + neo-XY, and the neo-XY is thought to be quite large [48] which can complicate a male genome assembly [37]. Further, we used only one mate-pair insert size that was rather small (~3kb), leading to little scaffolding of the many contigs. This is highlighted by the N50 contig size for the western pine beetle build being 4.5kb, which was only slightly smaller than the N50 scaffold size of 5kb (Table 1). The mountain pine beetle N50 contig size (7.4kb) was comparable to ours (4.5kb), yet the mountain pine beetle build used multiple mate-pair libraries which helped produce the reportedly longer scaffolds [37]. Additional longer mate-pair libraries in the future could greatly help scaffold the current genome assembly.

RADseq library preparation for western pine beetles, Entomocorticium *sp. B and* Ceratocystiopsis brevicomi

Approximately ~200 ng total DNA for each beetle and ~300ng for each fungal isolate was used at the start of the RADseq protocol. Each species was prepared separately and in their own set of libraries (8-12 individuals per library). We followed our own single restriction enzyme digest protocol which is similar to other single restriction enzyme digest protocols (e.g., Etter et al. 2011), yet replaces all column based cleanups with AMPure XP bead (Beckman Coulter, Brea CA) cleanups. We also used a slightly modified PE2 adapter (5'-

ATCGGAAGAGCGAGAACAA-3', 5'-

GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT- 3') which allows for combinatorial inline and third read barcoding of samples in a manner similar to double digest protocols [57]. In short, DNA was cleaned, digested with PstI (New England Biolabs), adapters with barcodes were ligated to the restriction cut sites, and samples were then pooled equally and sonicated using a Bioruptor (Diagenode Inc., Sparta, NJ). After shearing the DNA, it was blunt-end repaired, a 5′ A was ligated to the fragment, and then a small y-adapter (above) ligated to the A overhang on the fragment. The indexing read barcode and Illumina specific adapter sequences were then added to the fragments through PCR. PCR was done in 5 separate 20 ul reactions for each library using the following conditions: 98°C for 30s: 12-14X (98°C for 10s, 65°C for 30s, 72°C for 30s) with a final extension at 72°C for 5 minutes. An appropriate cycle number for each library was determined via qPCR and test amplifications over a range of cycles thereby ensuring sufficient amplification while keeping the cycle number as low as possible. Multiple reactions (4-6) for any one library were done to reduce to possibility of propagating any PCR error. Libraries from each species were pooled equivalently and sequenced on an Illumina HiSeq 2000 with 100 bp paired end reads at either the University of Utah Microarray and Genomic Analysis Core Facility (Salt Lake City, UT) or the Vincent J. Coates Genomics Sequencing Laboratory (Berkeley, CA).

RADseq read mapping and initial SNV identification

All raw reads were first processed using the process_radtags program from STACKS [58] version 1.20, using default settings and filtering out reads with low quality, allowing barcode recovery, and removing reads with uncalled bases. Paired reads that passed initial cleaning steps where then mapped to their respective draft genome using the BWA-MEM algorithm version

0.7.9 [59]. We then used samtools version 1.1 [60] and Picardtools version 1.84 to manipulate the sam/bam files and add read groups for downstream analyses. We removed PCR duplicates using samtools rmdup and generated general summary statistics of mapping results using samtools flagstat and GATK's DepthOfCoverage tool. To genotype individuals, we used GATK UnifiedGenotyper version 3.1-1 and genotyped all *Ceratocystiopsis*, *Entomocorticium*, and *Dendroctonus* samples in genus specific runs. Because of the larger number of *Dendroctonus* samples, we randomly assigned ~40 beetles to each genotyping run, and then merged the VCFs using GATK's CombineVariants tool.

Identifying high quality regions of the western pine beetle genome assembly and filtering SNVs

We first BLAST all western pine beetle scaffolds against the mountain pine beetle genome (*Dendroctonus ponderosae*) and identified western pine beetle scaffolds where the top hit was > 1kb. We then flagged for exclusion any scaffold that hit a mountain pine beetle X-linked scaffold [37]. Preliminary RADseq analyses suggested additional unidentified X chromosome scaffolds remained which influenced our analyses. To further identify putative X-linked scaffolds, we compared male/female RADseq coverage over all scaffolds, using read counts over RADtags from populations where we sequenced both males and females (SB, MI, SU, FL, RO, UT, PA). We used the GATK's DepthOfCoverage tool to estimate mean coverage for each individual over each RADtag, and then averaged across each sex and by scaffold. This allowed us to identify scaffolds that are putatively X-linked based on roughly double the coverage in females compared to males (Supplemental figure 1). Any scaffold where female coverage was >1.5× the male coverage was flagged for exclusion as putatively X chromosome (Supplemental figure 1). To confirm our coverage based methodology for identifying X-

chromosome scaffolds, we verified that the vast majority of scaffolds that had BLAST to the mountain pine beetle X chromosome showed excess coverage in females (Supplemental figure 1). After identifying scaffolds that BLAST to the mountain pine beetle genome, filtering out putatively X chromosome scaffolds, and removing small scaffolds without a PSTI restriction enzyme cutsite, we were left with 14,700 high quality scaffolds for further SNV identification

To identify high quality SNVs in our beetle samples, we genotyped all individuals using the GATK Unified Genotyper version 3.1-1. We first filtered the resulting VCF, with outgroups, using VCFtools version 0.1.12b [61]. We excluded sites that did not pass GATK's quality filtered out genotypes where the genotype quality was below 30. We initially sequenced 164 individuals, but during quality filtering, found four individuals had highly skewed allele depths (genome wide average for heterozygous positions should be ~50:50 reference allele:alternate allele). Suspiciously, they all had the same first read barcode, suggesting some contamination or barcoding issue. These samples were eliminated from further analyses. An additional four samples had very low sequencing coverage, and were also excluded, resulting in a total of 156 western pine beetles and three outgroups in the final analysis. To generate a dataset for initial phylogenetic analyses establishing the closest relative of the western pine beetle, we produced a dataset of just one high quality East and West western pine beetle along with all outgroups. For a dataset that included just the closest relative, *D. approximatus,* we thinned the full SNV dataset so that no SNVs were closer than 250bp. Further, we excluded genotype calls when depth < 5, maximum depth > 200 and more than 50% of individuals had missing data.

To generate a dataset for just western pine beetle population analysis, we returned to the initial VCF output from GATK and first excluded all outgroups. We further excluded any position that deviated significantly from Hardy-Weinberg proportions in any one of the 7

populations where we had data for seven or more individuals. This led us to exclude 959 additional positions. We then filtered to only include sites where 70% of individuals had a genotype call, and then filtered individual genotypes to exclude genotype calls when the minimum depth < 5, maximum depth > 200. We then only included biallelic sites and sites where the minor allele frequency was > 0.10.

To create a dataset to calculate divergence, diversity (π) and Tajima's D, we used the highest coverage East and West individuals so that we could confidently call both variant and invariant positions in a manner similar to the fungi (below). This included 28 individuals, 14 from East and 14 from West. To identify sites that could confidently be called across all individuals, we used the GATK Unified Genotyper version 3.1-1 and the output mode EMIT_ALL_SITES over genomic intervals found to have coverage in all individuals. Genotype calls were then made in all individuals over these regions as described above (excluding the minor allele frequency filter) and these genotype calls were concatenated.

Identifying high quality regions of the Entomocorticium *sp. B genome assembly and filtering SNVs*

Due to the repetitive nature of the *E.* sp. B genome (Table 1), and preliminary results that identified a few regions with highly concentrated heterozygosity indicative of collapsed paralogues in the genome build, we set out to mask these regions for downstream analyses. We re-mapped the short reads used to construct the reference genome back to our reference and compared the distribution of coverage over each position. Coverage was estimated using GATK's DepthOfCoverage tool. With median coverage estimated to be 68×, regions with reduced (< 30×) or excessive coverage (> 110×) were masked from SNV calling. Further,

lineage specific (*E*. sp. B_1, *E*. sp. B_2, and *E*. sp. B_3) paralogues were also identified in preliminary RADseq analyses and so to further exclude these regions from analyses, we masked regions where > 50% of calls in any one lineage were heterozygote calls. This removed an additional 73,616 bp of sequence (8% of the total). After these two genomic filters, only 3,656 heterozygote positions remained (0.01% of all genotype calls), which was comparable to analyses of the other fungal RADseq data and seemed sufficiently low.

We genotyped all individuals using the GATK Unified Genotyper version 3.1-1 with output mode EMIT_ALL_SITES. We then removed positions, using VCFtools version 0.1.12b [61] , that did not pass GATK's quality filters and/or where depth was < 10× for any one individual. Then, SNV calls with a genotype quality < 30 were changed to unknown, and we allowed for triallelic positions. After these filters, only a small percentage (0.01%) of all genotyping calls were still incorrect heterozygous calls, and those were all changed to missing data. We then concatenated all genotype calls for all individuals. Therefore, we had a genotype call for 315,655 variant + invariant positions scattered across the genome and used for subsequent phylogenetic analyses.

Because the phylogenetic comparison included sites that were present in all individuals, including divergent outgroups, there was the potential for biasing our population genetic analyses of *E*. sp. B toward conserved regions of the genome. To alleviate this issue, we refiltered the *E*. sp. B data after first removing outgroups. We used the filtering steps outlined above, yet restricted to sites that had at most two alleles. This resulted in 1,328,866 sites being called in all individuals. We then produced VCFs that included just variant and variant + invariant sites for downstream analyses.

Ceratocystiopsis brevicomi SNV filtering

The *C. brevicomi* genome was the most complete and had the fewest assembly errors and initial analyses suggested that additional masking of the genome was unnecessary. Therefore, we first genotyped all individuals using the GATK Unified Genotyper version 3.1-1 with output mode EMIT_ALL_SITES. We then removed the outgroup (*C. ranaculosus*) from the VCF produced by GATK and then removed positions, using VCFtools version 0.1.12b [61] , that did not pass GATK's quality filters and/or where depth was < 20× for any one individual. Then, SNV calls with a genotype quality < 30 were changed to unknown and only sites with no more than 2 alleles were kept. After these filters, only a small fraction (0.02%) of all genotyping calls were still incorrect heterozygous calls and those were all changed to missing data. In total, this lead to genotype call for 2,926,074 bases in every *C. brevicomi* individual. We then returned to the outgroup (*C. ranaculosus*) and filtered to only include positions genotyped (variant or invariant) in *C. brevicomi*, using the same depth and genotype quality filters described above but allowing for triallelic sites. VCFs of the variant + invariant, or just the variant positions were then used for downstream analyses.

Ponderosa pine population genetic data

To obtain ponderosa pine genetic data, we downloaded the microsatellite data for 7 SSRs originally described in Potter et al. [17]. We restricted our analyses to 13 sites that were in close geographic proximity to our beetle/fungal collection locations (Supplemental Table 1). Mean distance of these 13 ponderosa pine collection sites to our beetle/fungal sites was 28.9 K (min = 3 K, max = 66 K). For simplicity, we used our naming conventions for these sites (Supplemental Table 1).

Genetic analyses

To identify population genetic structure for the four species considered in our analysis, we used the programs STRUCTURE version 2.3.2.1[38] and ADMIXTURE version 1.23 [39] . We used STRUCTURE on the fungal and tree data since it has the ability to analyze both haploid (setting PLOIDY=1) and diploid data (PLOIDY =2). Due to computation limitations, we ran STRUCTURE analyses on 3,000 SNVs pulled at random, without replacement, from the two fungal datasets. For the tree and fungal analyses, the burnin was set at 20,000 and we allowed for 50,000 MCMC replicates after the burnin. We ran *K* values from one through eight and ran eight replicates per *K*. The best *K* for each species was then determined by visualizing the likelihoods in STRUCTURE HARVESTER [62] and based on the Evanno method [63]. Results for different runs for each species were averaged using CLUMPP 1.1.2 [64] and the posterior probabilities of assignment to each cluster were plotted using R version 3.1.1. For the beetle SNV data, we used the program ADMIXTURE version 1.23 [39] on 6,424 SNVs. We ran *K* values of one through 19 with 10-fold cross-validation. The *K* value with the lowest cross-validation standard error was considered the best *K* [39].

To further visualize population genetic structure we performed principal component analyses (PCA) for the four species. For our SNV data, we used Eigensoft [65], and 65,624, 6,874 and 6,424 SNVs for *E.* sp. B, *C. brevicomi*, and the western pine beetle, respectively. To perform PCA on the tree microsatellite data, we used GenAlEx [66, 67] and the covariance matrix of the allele frequencies with data standardization. All principle component analyses were visualized in R version 3.1.1.

To test for congruence among the genetic distance data matrixes of the four species, we used CADM [40] in the R package *ape* [68]. Genetic distance matrices used as input for analyses were generated for the tree, beetle, and *C. brevicomi* datasets using GenAlEx [66, 67]. Analyses were done using nine sites where we had data for all three species and included three East sites (MTC, FL, RO) and six West sites (SB, OL, CQ, LA, LF, DA). In CADM, 1,000 permutations were used to compute *a posteriori* tests and we used the Holm correction (default) to account for the multiple testing of distance matrices.

To infer the phylogenetic relationships among individuals for the two species of fungi (*E.* sp. B and *C. brevicomi*), we converted the VCF files that included both the variant + invariant positions into a PHYLIP format file. To construct maximum likelihood trees for the two species of fungi, we used RAxML version 8.0.23 [69] and a gtr + gamma model of nucleotide substitution with 200 bootstrap replicates. To infer the phylogenetic relationships among individual beetles, we first inferred the closest relative of the western pine beetle. We constructed preliminary unrooted trees using all outgroups and one East and West western pine beetle. We found strong support for *D. approximatus* as the closest relative (Supplemental Figure 2). We then used a concatenated matrix of thinned SNVs which were also converted into a PHYLIP format file. SNVs used to construct the tree were at a minimum 250bp away from each other. SNV thinning was done using VCFtools version 0.1.12b [61] and the --thin option. All resulting trees were visualized using FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

For all estimates of F_{st}, we calculated Weir and Cockerham's weighted F_{st} [43] using VCFtools version 0.1.12b [61]. To calculate absolute sequence divergence (D_{xy}) between East and West *C. brevicomi*, and between the three *E.* sp. B lineages, we used DNAsp version 5.10.1 [70]. We calculated D_{xy} in 2500 bp windows along a concatenated dataset and took the average

of all windows. To estimate nucleotide diversity (π) and Tajima's D, we used *PopGenome* [71]to determine per scaffold estimates and then took the average of these estimates. Comparisons of nucleotide diversity (π) and Tajima's D based on the average from the concatenation (i.e., DNAsp) to those that were independently estimated for each scaffold and then took the mean (*Popgenome*) were found to be very similar. To estimate nucleotide diversity (π) and D_{xy} for the western pine beetle data, we used the dataset for the 28 high coverage individuals and calculated these parameters in 5kb windows across the genome using a custom python script. We then took the average of these window-based estimates.

To test for recombination in the two fungal mutualists, *C. brevicomi* and *E.* sp. B, we used SplitsTree4 [72] and the PHI test (pairwised homoplasy index). For further conformation, we used the four-gamete test [73] as implemented in *PopGenome* [71].

Scanning electron microscope (SEM) imaging.

A ponderosa pine infested with western pine beetle was located in October of 2012 near the LF collection location (Supplemental Table 1). A portion of the outer bark was removed, and transported back to the University of Montana where the bark was fractured by hand to expose larvae and larval tunnels. Larval tunnels and bark were subsequently fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer and stored at 4° C prior to use. Samples were then dehydrated using a graded ethanol series and dried using a critical-point dryer (Balzars 030 critical-point dryer, BAL-TEC AG, Furstetum, Liechtenstein). Dried samples were then mounted on stubs and coated with gold-palladium using a Pelco Model 3 sputter-coater (Ted Pella, Inc.). All samples were visualized with a Hitachi S-4700 cold field emission SEM (Hitachi Inc.,

Pleasonton, California) and all sample processing took place at the University of Montana EMtrix facility.

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REFERENCES

1. Coyne, J.A. and H.A. Orr, *Speciation*. Vol. 37. 2004: Sinauer Associates Sunderland, MA.
2. Forister, M.L. and C.R. Feldman, *Phylogenetic cascades and the origins of tropical diversity.* Biotropica, 2011. **43**(3): p. 270-278.
3. Emerson, B.C. and N. Kolm, *Species diversity can drive speciation.* Nature, 2005. **434**(7036): p. 1015-1017.
4. Janz, N., S. Nylin, and N. Wahlberg, *Diversity begets diversity: host expansions and the diversification of plant-feeding insects.* Bmc Evolutionary Biology, 2006. **6**.
5. Forbes, A.A., et al., *Sequential Sympatric Speciation Across Trophic Levels.* Science, 2009. **323**(5915): p. 776-779.

6. Hood, G.R., et al., *Sequential divergence and the multiplicative origin of community diversity.* Proceedings of the National Academy of Sciences, 2015. **112**(44): p. E5980-E5989.

7. Mueller, U.G., et al., *The evolution of agriculture in insects.* Annual Review of Ecology Evolution and Systematics, 2005. **36**: p. 563-595.

8. Moran, N.A., *Symbiosis as an adaptive process and source of phenotypic complexity.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**: p. 8627-8633.

9. Wagner, S.M., et al., *Facultative endosymbionts mediate dietary breadth in a polyphagous herbivore.* Functional Ecology, 2015.

10. Clark, M.A., et al., *Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence.* Evolution, 2000. **54**(2): p. 517-525.

11. Conord, C., et al., *Long-term evolutionary stability of bacterial endosymbiosis in curculionoidea: Additional evidence of symbiont replacement in the dryophthoridae family.* Molecular Biology and Evolution, 2008. **25**(5): p. 859-868.

12. Noda, S., et al., *Cospeciation in the triplex symbiosis of termite gut protists (Pseudotrichonympha spp.), their hosts, and their bacterial endosymbionts.* Molecular Ecology, 2007. **16**(6): p. 1257-1266.

13. Six, D.L., *Ecological and evolutionary determinants of bark beetle—fungus symbioses.* Insects, 2012. **3**(1): p. 339-366.

14. Mehdiabadi, N.J., et al., *Symbiont fidelity and the origin of species in fungus-growing ants.* Nature Communications, 2012. **3**.

15. Aanen, D.K., et al., *The evolution of fungus-growing termites and their mutualistic fungal symbionts.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 14887-92.

16. Conkle, M.T. and W.B. Critchfield, *Genetic variation and hybridization of ponderosa pine*, in *Ponderosa pine: the species and its management*, D.M. Baumgartner and J.E. Lotan, Editors. 1988, Washington State University Press: Pullman, Washington. p. 27-43.

17. Potter, K., et al., *Nuclear genetic variation across the range of ponderosa pine (Pinus ponderosa): Phylogeographic, taxonomic and conservation implications.* Tree Genetics & Genomes, 2015. **11**(3): p. 1-23.

18. Latta, R.G. and J.B. Mitton, *Historical separation and present gene flow through a zone of secondary contact in ponderosa pine.* Evolution, 1999. **53**(3): p. 769-776.

19. Betancourt, J.L., T.R. Van Devender, and P.S. Martin, *Packrat middens: the last 40,000 years of biotic change.* 1990, Tucson, AZ: University of Arizona Press.

20. Potter, K.M., et al., *Mitochondrial DNA Haplotype Distribution Patterns in Pinus Ponderosa (Pinaceae): Range-Wide Evolutionary History and Implications for Conservation.* American Journal of Botany, 2013. **100**(8): p. 1562-1579.

21. Roberts, D.R. and A. Hamann, *Glacial refugia and modern genetic diversity of 22 western North American tree species.* Proceedings of the Royal Society B-Biological Sciences, 2015. **282**(1804).

22. Smith, R.H., *Monoterpenes of ponderosa pine xylem resin in western United States*. 1977, USDA. p. 1-48.

23. Miller, J.M. and F.P. Keen, *Biology and control of the western pine beetle: a summary of the first 50 years of research*. 1960, Washington, DC: U.S. Department of Agriculture.

24. Wood, S.L., *The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae).* Great Basin Naturalist, 1982. **6**: p. 1-1359.

25. Byers, J.A., *Host-tree chemistry affecting colonization in bark beetles*, in *Chemical ecology of insects 2*. 1995, Springer. p. 154-213.

26. Kelley, S.T., J.B. Mitton, and T.D. Paine, *Strong differentiation in mitochondrial DNA of Dendroctonus brevicomis (Coleoptera : Scolytidae) on different subspecies of ponderosa pine.* Annals of the Entomological Society of America, 1999. **92**(2): p. 193-197.

27. Kelley, S.T. and B.D. Farrell, *Is specialization a dead end? The phylogeny of host use in Dendroctonus bark beetles (Scolytidae).* Evolution, 1998. **52**(6): p. 1731-1743.

28. Hsiau, P.T.W. and T.C. Harrington, *Ceratocystiopsis brevicomi sp nov, a mycangial fungus from Dendroctonus brevicomis (Coleoptera: Scolytidae).* Mycologia, 1997. **89**(4): p. 661-669.

29. Hsiau, P.T.W. and T.C. Harrington, *Phylogenetics and adaptations of basidiomycetous fungi fed upon by bark beetles (Coleoptera : Scolytidae).* Symbiosis, 2003. **34**(2): p. 111-131.

30. Bracewell, R.R. and D.L. Six, *Experimental evidence of bark beetle adaptation to a fungal symbiont.* Ecology and Evolution, 2015. **5**(21): p. 5109-5119.

31. Paine, T.D. and M.C. Birch, *Acquisition and Maintenance of Mycangial Fungi by Dendroctonus-Brevicomis Leconte (Coleoptera, Scolytidae).* Environmental Entomology, 1983. **12**(5): p. 1384-1386.

32. Harrington, T.C., *Ecology and evolution of mycophagous bark beetles and their fungal partners*, in *Ecological and Evolutionary Advances in Insect-Fungal Associations*, F.E. Vega and M. Blackwell, Editors. 2005, Oxford University Press. p. 257-291.

33. Bracewell, R.R. and D.L. Six, *Broadscale Specificity in a Bark Beetle-Fungal Symbiosis: a Spatio-temporal Analysis of the Mycangial Fungi of the Western Pine Beetle.* Microbial Ecology, 2014. **68**(4): p. 859-870.
34. Valiev, A., Z.B. Ogel, and K.D. Klepzig, *Analysis of cellulase and polyphenol oxidase production by southern pine beetle associated fungi.* Symbiosis, 2009. **49**(1): p. 37-42.
35. Gnerre, S., et al., *High-quality draft assemblies of mammalian genomes from massively parallel sequence data.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(4): p. 1513-1518.
36. Parra, G., K. Bradnam, and I. Korf, *CEGMA: a pipeline to accurately annotate core genes in eukaryotic genornes.* Bioinformatics, 2007. **23**(9): p. 1061-1067.
37. Keeling, C.I., et al., *Draft genome of the mountain pine beetle, Dendroctonus ponderosae Hopkins, a major forest pest.* Genome Biology, 2013. **14**(3).
38. Pritchard, J.K., M. Stephens, and P. Donnelly, *Inference of population structure using multilocus genotype data.* Genetics, 2000. **155**(2): p. 945-959.
39. Alexander, D.H., J. Novembre, and K. Lange, *Fast model-based estimation of ancestry in unrelated individuals.* Genome Research, 2009. **19**(9): p. 1655-1664.
40. Legendre, P. and F.J. Lapointe, *Assessing congruence among distance matrices: Single-malt Scotch whiskies revisited.* Australian & New Zealand Journal of Statistics, 2004. **46**(4): p. 615-629.
41. Tsui, C.K.M., et al., *Population Structure of Mountain Pine Beetle Symbiont Leptographium longiclavatum and the Implication on the Multipartite Beetle-Fungi Relationships.* Plos One, 2014. **9**(8).

42. Roe, A.D., et al., *Comparative phylogeography, genetic differentiation and contrasting reproductive modes in three fungal symbionts of a multipartite bark beetle symbiosis.* Molecular Ecology, 2011. **20**(3): p. 584-600.

43. Weir, B.S. and C.C. Cockerham, *Estimating F-Statistics for the Analysis of Population-Structure.* Evolution, 1984. **38**(6): p. 1358-1370.

44. Mikheyev, A.S., U.G. Mueller, and P. Abbot, *Cryptic sex and many-to-one colevolution in the fungus-growing ant symbiosis.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(28): p. 10702-10706.

45. Tsui, C.K.M., et al., *Population structure and migration pattern of a conifer pathogen, Grosmannia clavigera, as influenced by its symbiont, the mountain pine beetle.* Molecular Ecology, 2012. **21**(1): p. 71-86.

46. Whitney, H.S., R.J. Bandoni, and F. Oberwinkler, *Entomocorticium-Dendroctoni Gen Et Sp-Nov (Basidiomycotina), a Possible Nutritional Symbiote of the Mountain Pine-Beetle in Lodgepole Pine in British-Columbia.* Canadian Journal of Botany-Revue Canadienne De Botanique, 1987. **65**(1): p. 95-102.

47. Schultz, S.T. and M. Lynch, *Mutation and extinction: The role of variable mutational effects, synergistic epistasis, beneficial mutations, and degree of outcrossing.* Evolution, 1997. **51**(5): p. 1363-1371.

48. Lanier, G.N., J.P. Hendrichs, and J.E. Flores, *Biosystematics of the Dendroctonus-Frontalis (Coleoptera, Scolytidae) Complex.* Annals of the Entomological Society of America, 1988. **81**(3): p. 403-418.

49. Feder, J.L. and A.A. Forbes, *Sequential speciation and the diversity of parasitic insects.* Ecological Entomology, 2010. **35**: p. 67-76.

50. Byers, J., *Male-specific conversion of the host plant compound, myrcene, to the pheromone, (+)-ipsdienol, in the bark beetle,Dendroctonus brevicomis.* Journal of Chemical Ecology, 1982. **8**(2): p. 363-371.

51. Hofstetter, R.W., et al., *Synergistic effects of alpha-pinene and exo-brevicomin on pine bark beetles and associated insects in Arizona.* Journal of Applied Entomology, 2008. **132**(5): p. 387-397.

52. Alamouti, S.M., et al., *Gene genealogies reveal cryptic species and host preferences for the pine fungal pathogen Grosmannia clavigera.* Mol Ecol, 2011. **20**(12): p. 2581-602.

53. Six, D.L. and T.D. Paine, *Phylogenetic comparison of ascomycete mycangial fungi and Dendroctonus bark beetles (Coleoptera : Scolytidae).* Annals of the Entomological Society of America, 1999. **92**(2): p. 159-166.

54. Brown, A.M.V., et al., *Population genomics of a symbiont in the early stages of a pest invasion.* Molecular Ecology, 2014. **23**(6): p. 1516-1530.

55. Zimin, A.V., et al., *The MaSuRCA genome assembler.* Bioinformatics, 2013. **29**(21): p. 2669-2677.

56. Leggett, R.M., et al., *NextClip: an analysis and read preparation tool for Nextera Long Mate Pair libraries.* Bioinformatics, 2014. **30**(4): p. 566-568.

57. Peterson, B.K., et al., *Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species.* Plos One, 2012. **7**(5).

58. Catchen, J., et al., *Stacks: an analysis tool set for population genomics.* Molecular Ecology, 2013. **22**(11): p. 3124-3140.

59. Li, H. and R. Durbin, *Fast and accurate short read alignment with Burrows-Wheeler transform.* Bioinformatics, 2009. **25**(14): p. 1754-1760.

60. Li, H., et al., *The Sequence Alignment/Map format and SAMtools.* Bioinformatics, 2009. **25**(16): p. 2078-2079.

61. Danecek, P., et al., *The variant call format and VCFtools.* Bioinformatics, 2011. **27**(15): p. 2156-2158.

62. Earl, D.A. and B.M. Vonholdt, *STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method.* Conservation Genetics Resources, 2012. **4**(2): p. 359-361.

63. Evanno, G., S. Regnaut, and J. Goudet, *Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study.* Molecular Ecology, 2005. **14**(8): p. 2611-2620.

64. Jakobsson, M. and N.A. Rosenberg, *CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure.* Bioinformatics, 2007. **23**(14): p. 1801-1806.

65. Price, A.L., et al., *Principal components analysis corrects for stratification in genome-wide association studies.* Nature Genetics, 2006. **38**(8): p. 904-909.

66. Peakall, R. and P.E. Smouse, *GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update.* Bioinformatics, 2012. **28**(19): p. 2537-2539.

67. Peakall, R. and P.E. Smouse, *GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research.* Molecular Ecology Notes, 2006. **6**(1): p. 288-295.

68. Paradis, E., J. Claude, and K. Strimmer, *APE: Analyses of Phylogenetics and Evolution in R language.* Bioinformatics, 2004. **20**(2): p. 289-290.

69. Stamatakis, A., *RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies.* Bioinformatics, 2014. **30**(9): p. 1312-1313.

70. Librado, P. and J. Rozas, *DnaSP v5: a software for comprehensive analysis of DNA polymorphism data.* Bioinformatics, 2009. **25**(11): p. 1451-1452.

71. Pfeifer, B., et al., *PopGenome: An Efficient Swiss Army Knife for Population Genomic Analyses in R.* Molecular Biology and Evolution, 2014. **31**(7): p. 1929-1936.

72. Huson, D.H. and D. Bryant, *Application of phylogenetic networks in evolutionary studies.* Molecular Biology and Evolution, 2006. **23**(2): p. 254-267.

73. Hudson, R.R. and N.L. Kaplan, *Statistical Properties of the Number of Recombination Events in the History of a Sample of DNA-Sequences.* Genetics, 1985. **111**(1): p. 147-164.

Table 1. Genome assembly statistics for the beetle (*Dendroctonus brevicomis*) the basidiomycete (*Entomocorticium* sp. B) and the ascomycete (*Ceratocystiopsis brevicomi*).

Organism	Number of scaffolds	Total length with gaps	N50 contig length (kb)	N50 scaffold length (kb)	Maximum scaffold length	GC content	Estimated genome size	Estimated repetitiveness of genome
beetle (*Dendroctonus brevicomis*)	35,469	129,552,754	4.5	5	540,064	37%	191,539,754	28%
basidiomycete (*Entomocorticium sp. B*)	1,248	35,715,059	36.9	54	302,230	50%	64,149,417	54%
ascomycete (*Ceratocystiopsis brevicomi*)	198	20,626,708	22.8	1,358	3,492,413	57%	29,374,628	40%

Table 2. Nucleotide diversity and Tajima's D estimates for East and West.

Organism	Region (East or West)	Number of individuals	Nucleotide diversity (π)	Tajima's D
Basidiomycete (*E.* sp. B)	West (B_1)	17	0.00022	-0.666
	West (B_2)	8	0.00014	-0.542
	East (B_3)	11	0.00016	-0.599
Ascomycete (*C. brevicomi*)	West	18	0.00401	-1.160
	East	4	0.00078	*
Beetle (*D. brevicomis*)	West	14	0.00805	-0.599
	East	14	0.00577	-0.450

Supplemental Table 1. Summary of collection locations and number of individuals genotyped per area. Beetles were collected using funnel traps baited with attractants (funnel), were extracted from trees (new attack) or reared out of bark from infested trees. For each species, per location, the type of genetic data used is presented and the number of individuals from the site is shown in parentheses.

Beetle collector	Beetle collection method	Location	Nearest town	Identifier	*E*. sp B	*C. brevicomi*	*D. brevicomis*	*P. ponderosa**
Ryan Bracewell	funnel	33° 28' N, 109° 22' W	Morenci, AZ	AP	RADseq(2)	NA	RADseq(5)	microsatellite(70)
Glenn Kohler	reared from bark	47° 16' N, 117° 34' W	Cheney, WA	CH	RADseq(2)	RADseq(1)	RADseq(5)	NA
Ryan Bracewell	funnel	42° 46' N, 121° 44' W	Chiloquin, OR	CQ	RADseq(2)	RADseq(1)	RADseq(4)	microsatellite(30)
Ryan Bracewell	reared from bark	46° 04' N, 114° 14' W	Darby, MT	DA	RADseq(1)	RADseq(1)	RADseq(5)	microsatellite(30)
Darci Carlson	funnel	47° 45' N, 120° 25' W	Entiat, WA	EN	RADseq(1)	NA	RADseq(5)	microsatellite(30)
Rich Hofstetter	funnel	35° 10' N, 111° 45' W	Flagstaff, AZ	FL	RADseq(2)	RADseq(1)	RADseq(8)	microsatellite(29)
Andy Graves	funnel	32° 53' N, 107° 45' W	Gila, NM	GI	RADseq(1)	NA	RADseq(3)	NA
Ryan Bracewell	funnel	45° 54' N, 120° 42' W	Goldendale, WA	GO	RADseq(1)	RADseq(1)	RADseq(5)	NA
Ryan Bracewell	funnel	48° 34' N, 118° 18' W	Kettle Falls, WA	KE	RADseq(1)	RADseq(1)	RADseq(5)	NA
Lia Spiegel	funnel	45° 19' N, 118° 19' W	La Grande, OR	LA	RADseq(1)	RADseq(1)	RADseq(5)	microsatellite(30)
Diana Six	funnel	46° 53' N, 113° 28' W	Greenough, MT	LF	RADseq(2)	RADseq(1)	RADseq(5)	microsatellite(26)
Ryan Bracewell	reared from bark	44° 06' N, 115° 21' W	Lowman, ID	LO	RADseq(1)	RADseq(1)	RADseq(5)	NA
Cynthia Snyder	funnel	41° 17' N, 122° 03' W	McCloud, CA	MC	RADseq(1)	NA	RADseq(5)	NA
Ryan Bracewell	funnel	46° 49' N, 114° 08' W	Missoula, MT	MI	RADseq(1)	RADseq(1)	RADseq(7)	NA
Ryan Bracewell	funnel	36° 18' N, 115° 40' W	Mt. Charleston, NV	MTC	RADseq(1)	RADseq(1)	RADseq(4)	microsatellite(30)
Danny Cluck	funnel	40° 41' N, 121° 13' W	Old Station, CA	OL	RADseq(1)	RADseq(1)	RADseq(4)	microsatellite(34)
Ryan Bracewell	funnel	37° 21' N, 107° 19' W	Piedra, CO	PA	RADseq(2)	NA	RADseq(8)	microsatellite(29)
Steve McKelvey	funnel	38° 44' N, 120° 44' W	Placerville, CA	PL	RADseq(1)	RADseq(1)	RADseq(5)	NA
Ryan Bracewell	reared from bark	44° 27' N, 120° 26' W	Prineville, OR	PV	RADseq(1)	RADseq(1)	RADseq(5)	NA
Ryan Bracewell	new attack	33° 28' N, 105° 44' W	Ruidoso, NM	RO	RADseq(1)	RADseq(1)	RADseq(8)	microsatellite(30)
Tom Coleman	funnel and bark	34° 10' N, 116° 55' W	San Bernardino, CA	SB	RADseq(2)	RADseq(1)	RADseq(7)	microsatellite(30)
Beverly Bulaon	funnel	37° 59' N, 120° 05' W	Tuolumne City, CA	SierraI	RADseq(1)	RADseq(1)	RADseq(2)	NA
Beverly Bulaon	funnel	38° 13' N, 120° 22' W	Avery, CA	SierraII	RADseq(1)	RADseq(1)	RADseq(5)	NA
Beverly Bulaon	funnel	37° 02' N, 119° 14' W	Bretz Mill, CA	SierraIII	RADseq(1)	NA	RADseq(5)	microsatellite(30)
Ryan Bracewell	funnel/bark	49° 41' N, 119° 46' W	Summerland, BC	SU	RADseq(1)	RADseq(1)	RADseq(8)	NA
Darci Carlson	funnel	48° 16' N, 120° 11' W	Twisp, WA	TW	RADseq(1)	RADseq(1)	RADseq(5)	NA
Ryan Bracewell	funnel	37° 35' N, 112° 15' W	Tropic, UT	UT	RADseq(1)	RADseq(1)	RADseq(8)	NA
Ryan Bracewell	reared from bark	44° 53' N, 115° 42' W	Yellow Pine, ID	YE	RADseq(1)	RADseq(1)	RADseq(5)	NA
Rich Hofstetter	funnel	34° 32' N, 112° 32' W	Prescott, AZ	PR	RADseq(1)	NA	RADseq(5)	NA

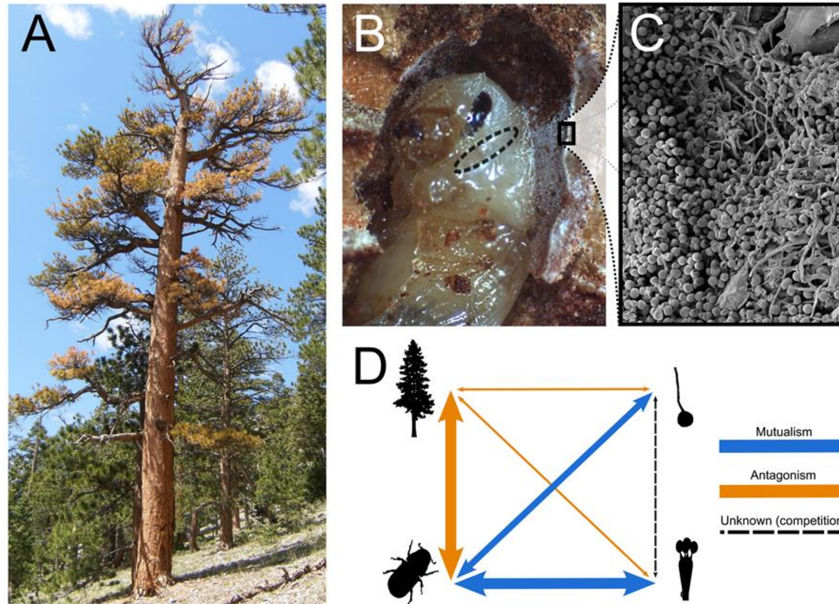


Figure 1. The ponderosa pine-western pine beetle-fungal symbiont interaction. A) The ponderosa pine is an iconic tree widely distributed across the western U.S. B) The western pine beetle attacks and kills ponderosa pine by constructing tunnels and reproducing in the phloem. After successful tree colonization and inoculation of fungal symbionts, developing larvae leave the phloem and tunnel into the nutrient poor outer bark where they feed heavily on symbiotic fungi that provide critical nutrients to the developing insect. The beetle-fungal symbiosis is maintained via an exoskeletal structure in the female (mycangium; its location highlighted with an ellipse) that harbors glands and excretes unknown substances thought to nurture and promote specificity [30]. After pupation, adult beetles incorporate spores into the mycangium for transport to the next host tree. C) Shown is an SEM image (see Supplemental figure 3 for additional images) of symbiotic fungi and spores (likely the basidiomycete, *E*. sp. B) lining the pupal chamber. D) Schematic of the complexity of the tree-beetle-fungi interaction, ranging from antagonism to mutualism between the four organisms. Arrow widths scaled to represent the strength of the interaction.

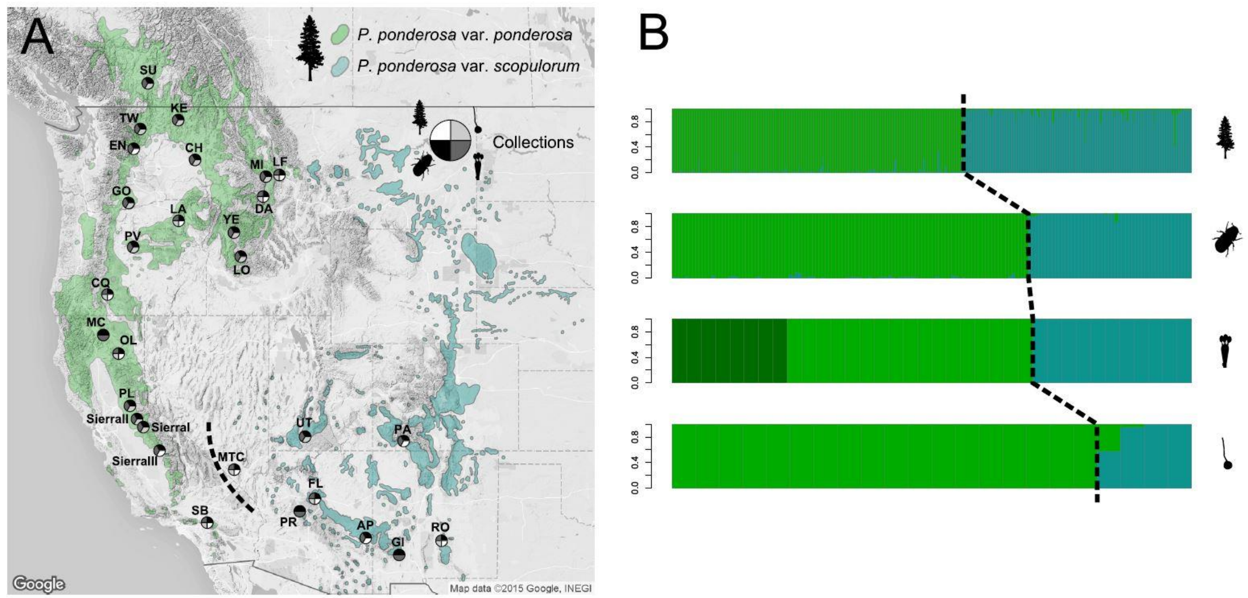


Figure 2. Geographic distribution and codifferentiation in the tree-beetle-fungi system. A) Ponderosa pine is broadly distributed across the western U.S. and is comprised of two subspecies (var. *ponderosa* and var. *scopulorum*) thought to have formed in isolation in southern refugia during the Pleistocene. The distribution of the beetle currently follows its primary host tree, except where absent in the central and northern portion of the *P. ponderosa* var. *scopulorum* range. Tree, beetle and fungal collection locations are shown and when present at a location are represented in the pie chart. B) STRUCTURE and ADMIXTURE results for the tree, beetle, and two symbiotic fungi and the posterior probability of assignment for each individual (vertical bar) to the optimal number of genetic clusters (*K*) for each species.

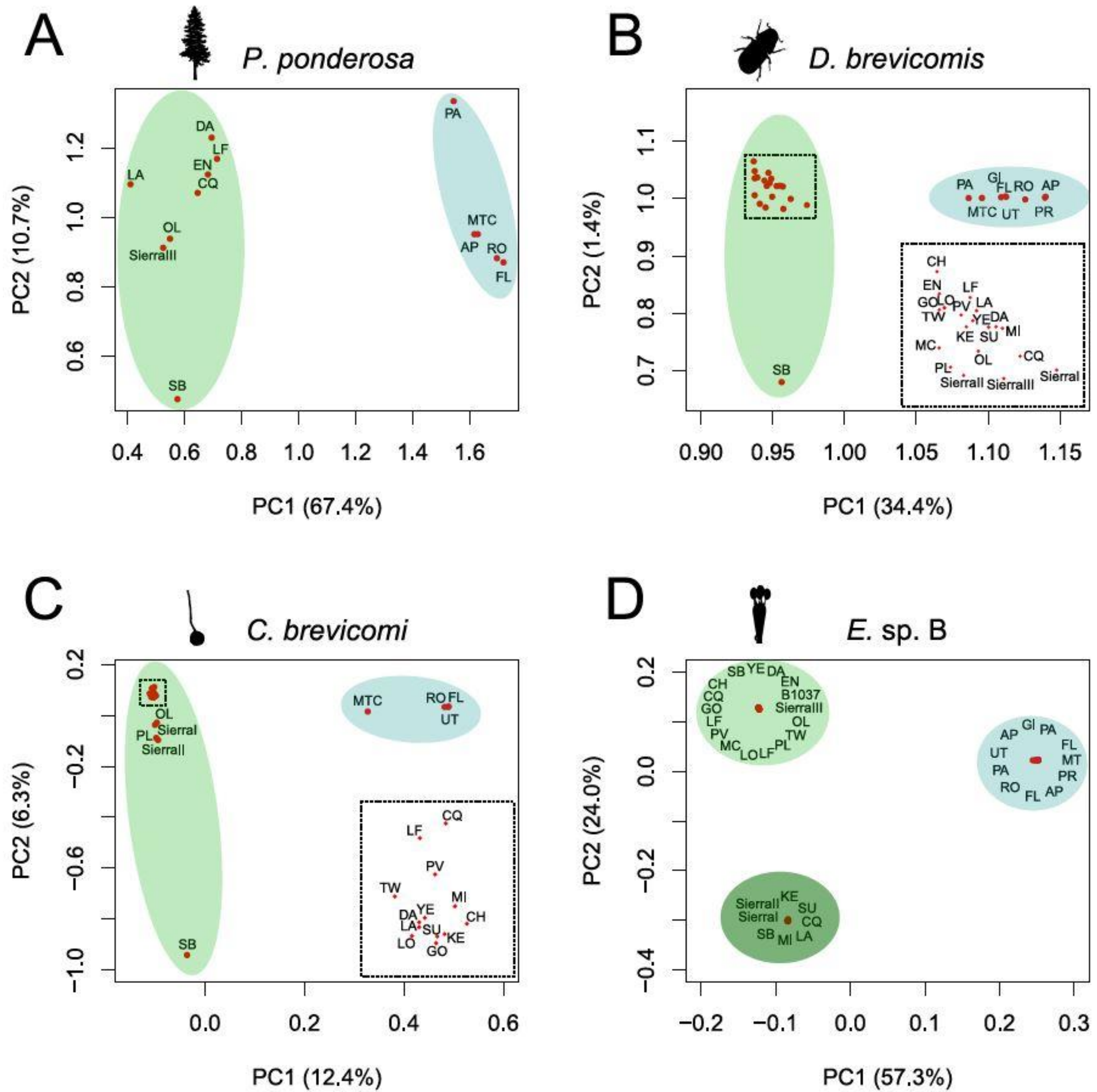


Figure 3. Fine-scale population structuring and relationships among sites. Results of principal component analyses for the A) tree, B) beetle, and symbiotic C) ascomycete and D) basidiomycete. Highlighted in green are West sites, and in blue are East sites. Colors correspond to those used in Fig.1B. Dense clusters of points highlighted in boxes are expanded in the bottom right.

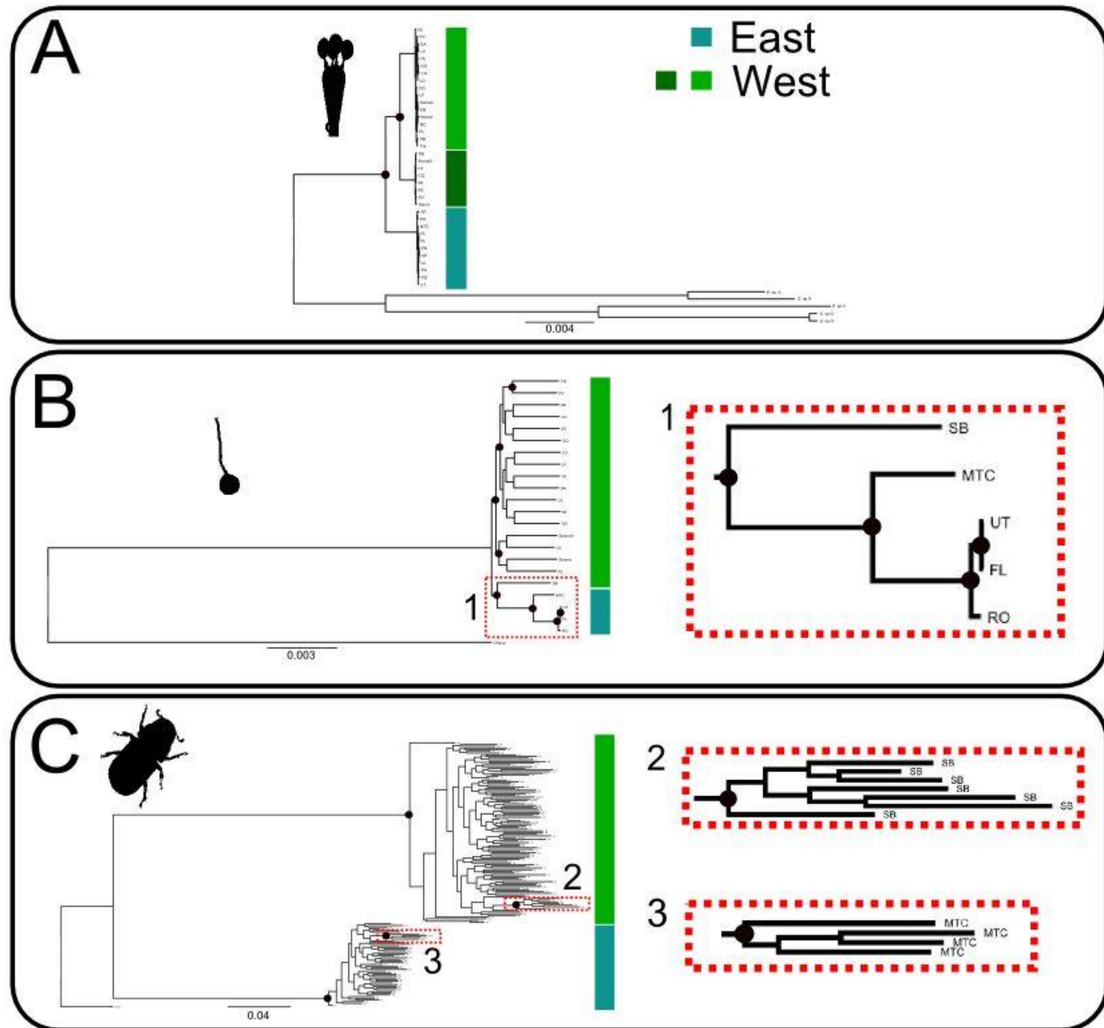
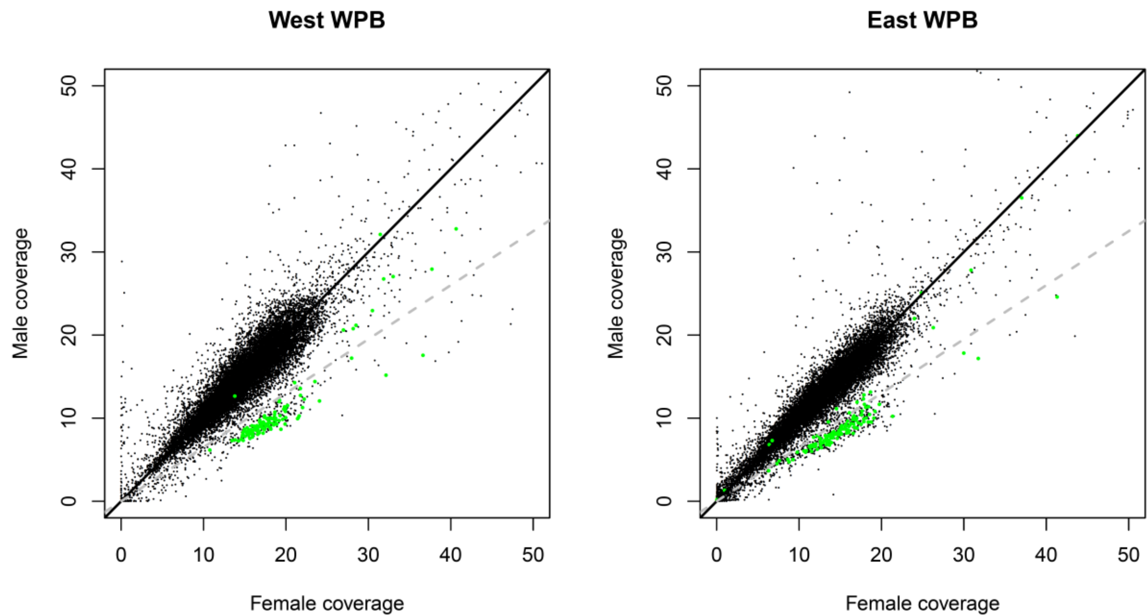
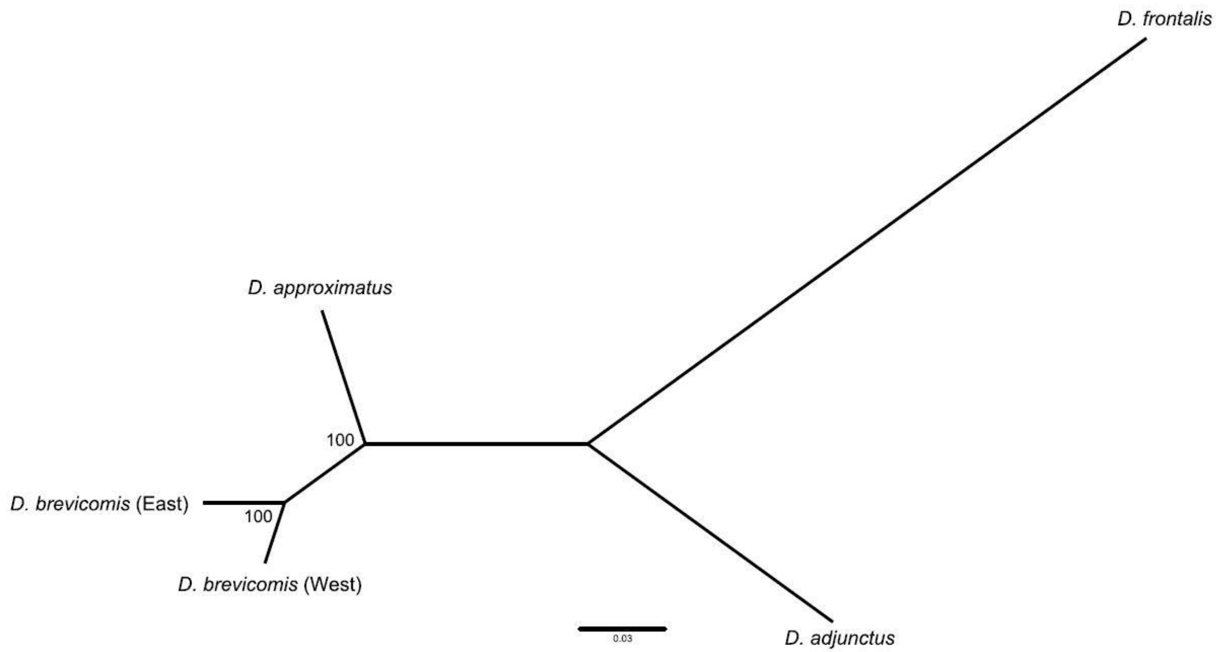


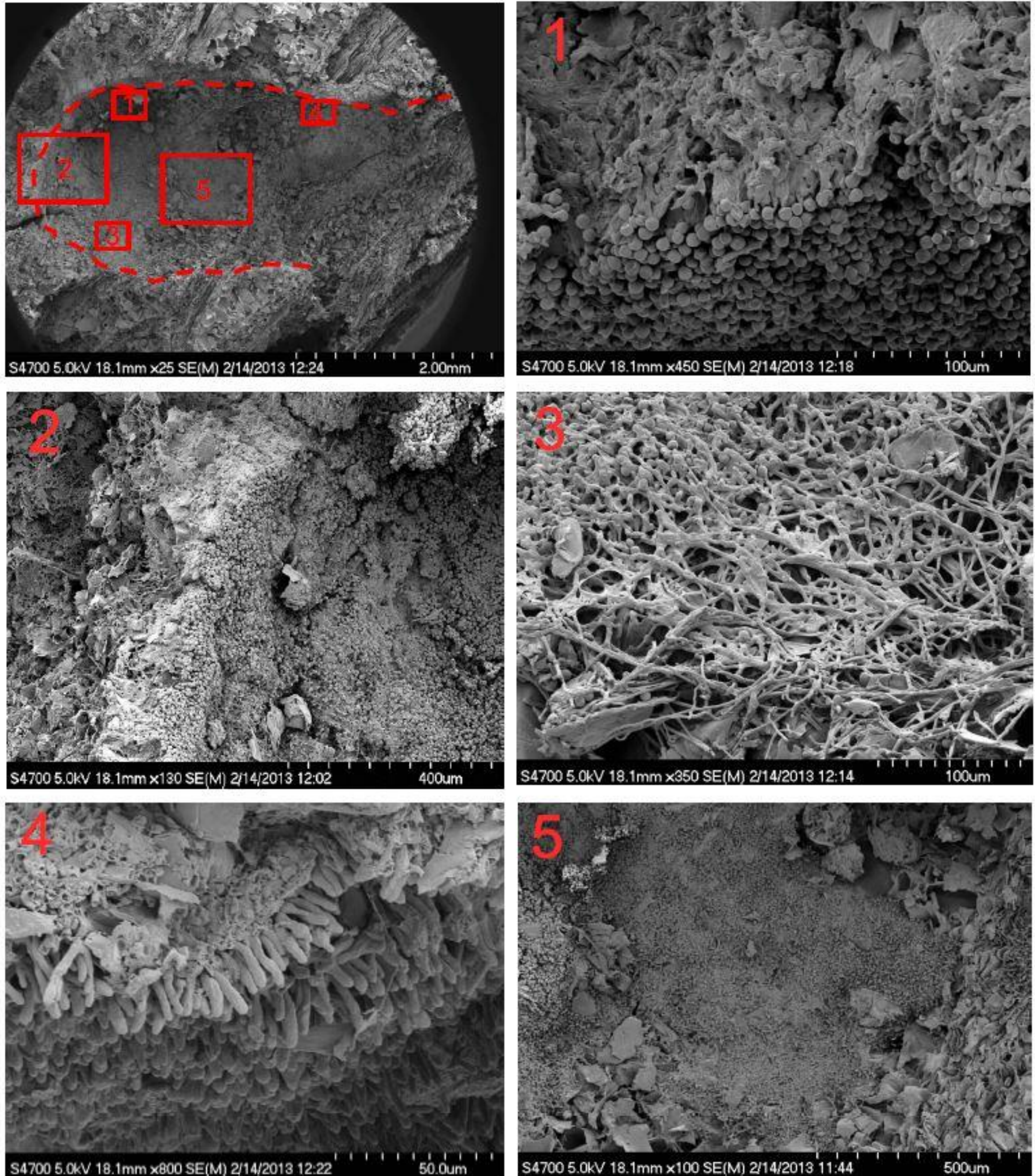
Figure 4. Phylogenetic relationships among individuals. Rooted maximum likelihood trees (RAxML, GTR+ gamma) for the A) basidiomycete, B) ascomycete, and C) beetle. For the beetle, only the most closely related outgroup (*D. approximatus*) is shown. Bootstrap support ≥ 80 shown with a dot. A total of 315,655 and 2,926,074 bp of genomic sequence (both variant and invariant positions) was used to infer the relationships among the basidiomycete and ascomycete individuals, respectively. A total of 16,112 concatenated SNVs were used to infer the relationships among beetles. Important portions of the trees are highlighted, numbered and shown at right.



Supplemental figure 1. Identifying sex-linked scaffolds in the western pine beetle (WPB) genome. Shown is the standardized sequencing coverage of male and female RADtags (DNA sequence immediately adjacent a restriction enzyme cutsite) over scaffolds in the western pine beetle genome build. Each scaffold shown as a point. Green points are scaffolds with significant BLAST hits to putative X chromosome scaffolds in the mountain pine beetle. Scaffolds above the hashed grey line were considered to be autosomal and used for SNV detection and further analysis



Supplemental figure 2. Phylogenetic relationships among closely related prothoracic mycangium-bearing *Dendroctonus* species. Unrooted maximum likelihood (RAxML, GTR + gamma) tree of concatenated SNVs (33,808) from East and West western pine beetles and three close relatives.



Supplemental figure 3. Scanning electron microscope (SEM) images of a western pine beetle larval tunnel (with larva removed) and symbiotic fungal growth. In the first SEM image, the larval tunnel (dashed outline) and the location of the magnified images shown in subsequent images (1-5) are shown.