

*Full Length Research Paper*

# Interactions between multiple fungi isolated from two bark beetles, *Dendroctonus brevicomis* and *Dendroctonus frontalis* (Coleoptera: Curculionidae)

Thomas S. Davis<sup>1</sup>, Richard W. Hofstetter<sup>1</sup>, Kier D. Klepzig<sup>2</sup>, Jeffrey T. Foster<sup>3</sup> and Paul Keim<sup>3</sup>

<sup>1</sup>Northern Arizona University, School of Forestry, P. O. BOX 15018, Flagstaff, Arizona, USA, 86011.

<sup>2</sup>USDA Forest Service, Southern Research Station, Pineville, Louisiana, USA.

<sup>3</sup>Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, Arizona, USA.

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Antagonism between the fungal symbionts of bark beetles may represent a biologically significant interaction when multiple beetle species co-occur in a host tree. Since high density bark beetle populations rapidly and dramatically shift forest characteristics, patterns of competition between the obligate fungal associates of sympatric bark beetle species may have broad ecological effects. Primary and competitive resource acquisition between allopatric and sympatric isolates of mutualist fungi associated with the bark beetles *Dendroctonus frontalis* and *Dendroctonus brevicomis* were investigated. Growth assays at multiple temperatures suggest that primary resource acquisition by fungi growing in the absence of competitors varies regionally, and that optimal growth rate is likely to correspond to average summertime maximum temperatures. In competition assays, interactions were asymmetric between fungi isolated from sympatric beetle populations and fungi isolated from allopatric beetle populations: sympatric isolates out-competed allopatric isolates. However, competition between fungi from beetle populations in sympatry was found to be equal. These studies are the first to investigate interactions between the mycangial fungi of multiple *Dendroctonus* species, and the results suggest that competition is likely to occur when the mycangial fungi of multiple beetle species occur together.

**Key words:** Allopatric, competition, coexistence, mutualism, mutualist, mycangial fungi, sympatric.

## INTRODUCTION

In many ecosystems, symbiotic associations are ubiquitous (Bronstein, 1994). For species that co-evolve with symbionts, interaction with symbionts is strongly correlated with population performance (e.g. fig/fig wasp, yucca/ yucca moth, ants/myrmecophytes, and beetle/fungus mutualisms; Bronstein, 1992; Huth and Pellmyr, 1997; Klepzig et al., 2001; Palmer et al., 2003); however, little research has investigated competitive symmetry among species with multiple symbionts (Palmer et al., 2003). *Dendroctonus* beetles (Coleoptera: Curculionidae) represent a useful system for studying

interactions between symbiotic species (Six and Klepzig, 2004). *Dendroctonus* beetles associate with an extensive community of microorganisms, including mites, nematodes, fungi, yeasts, and bacteria (Whitney, 1982; Klepzig et al., 2001; Kenis et al., 2004; Kirisits, 2004; Scott et al., 2008). The composition and abundance of these microbial communities considerably impact beetle population dynamics (Bridges, 1983; Paine et al., 1997; Hofstetter et al., 2006), and previous studies have cited a need for investigating interactions among the microbial associates of *Dendroctonus* beetles (Klepzig and Wilkens, 1997; Harrington, 2005).

*Dendroctonus* species construct tunnels in the vascular tissue of host conifers in order to lay eggs (Wood, 1982). During excavation, tunnels are inoculated with fungal symbionts that grow throughout host tissues and deve-

\*Corresponding author. E-mail: [tsd3@nau.edu](mailto:tsd3@nau.edu). Tel: 928-523-6452. Fax: 928-523-1080.

loping beetle larvae feed upon them (Harrington, 2005). In most cases *Dendroctonus* species are allopatric or colonize different tree species (Wood, 1982; Lieutier et al., 2004), so the opportunity for antagonism among fungal symbionts are avoided during larval development (Schlyter and Anderbrandt, 1993). However, in the ponderosa pine (*Pinus ponderosa* var *brachyptera*) forests of Southwestern North America, *Dendroctonus brevicomis* LeConte and *Dendroctonus frontalis* Zimmerman have been reported to co-colonize tissues of ponderosa pine (*Pinus ponderosa*) and with no apparent negative impacts on the fitness or fecundity of either beetle species (Davis and Hofstetter, 2009).

For *D. brevicomis* and *D. frontalis*, fungal symbionts in the genera *Ceratocystiopsis* and *Entomocorticium* confer important benefits to developing larvae (Klepzig and Wilkens, 1997; Hsiau and Harrington, 1997). For example, the presence of these filamentous fungi in feeding chambers is correlated with adult beetle size, fecundity, and nitrogen content (Bridges, 1983; Coppedge et al., 1995; Ayres et al., 2000). Also, beetles have evolved glandular structures (termed 'mycangia') that are used for transporting these fungi between host trees (Barras and Perry, 1971; Hsiau and Harrington, 1997; Yuceer et al., 2010). Due to the prevalence of association and interdependence of mycangial fungi and multiple *Dendroctonus* species, the beetle-fungal relationship is often considered a mutualism (Six, 2003). However, the sign (+, -) of interaction between multiple fungal symbionts of sympatric *Dendroctonus* species is unknown. But, interactions between fungal species that co-inhabit a niche have been shown to be antagonistic in many natural systems (Klepzig and Wilkens, 1997; Yuen et al., 1999; Murphy and Mitchell, 2001; Klepzig, 2006; Boddy, 2007; Licyayo et al., 2007). Uncolonized pine vascular tissue is a limiting resource for mycangial fungi; this should create an interface for antagonism between fungal mutualists when multiple beetle species co-colonize a host. Among sympatric *Dendroctonus* species that co-inhabit host tissue, competition between fungal symbionts of beetles may represent an interaction that limits beetle fitness or population growth.

Here, the authors report on patterns of primary and competitive resource acquisition by mycangial fungi of sympatric and allopatric *Dendroctonus* beetles. Throughout this report, "primary resource acquisition" is defined as the rate at which fungal isolates acquired resource area in the absence of competitors. "Competitive resource acquisition" is defined as the average proportion of trials in which individual fungal isolates acquired resource space that was occupied by another fungal isolate. The authors experimentally investigated the effects of temperature and biotic interactions on growth patterns of 33 isolates of mutualistic mycangial fungi associated with *D. brevicomis* and *D. frontalis*, from both sympatric and allopatric beetle populations. We ask two questions: (1) Does primary resource acquisition by mycangial isolates vary by fungal species, beetle species, or

beetle populations? (2) Does the symmetry of competitive interactions by mycangial isolates vary with sympatry or allopatry of beetle populations?

## MATERIALS AND METHODS

### Beetle collection and acquisition of fungal isolates

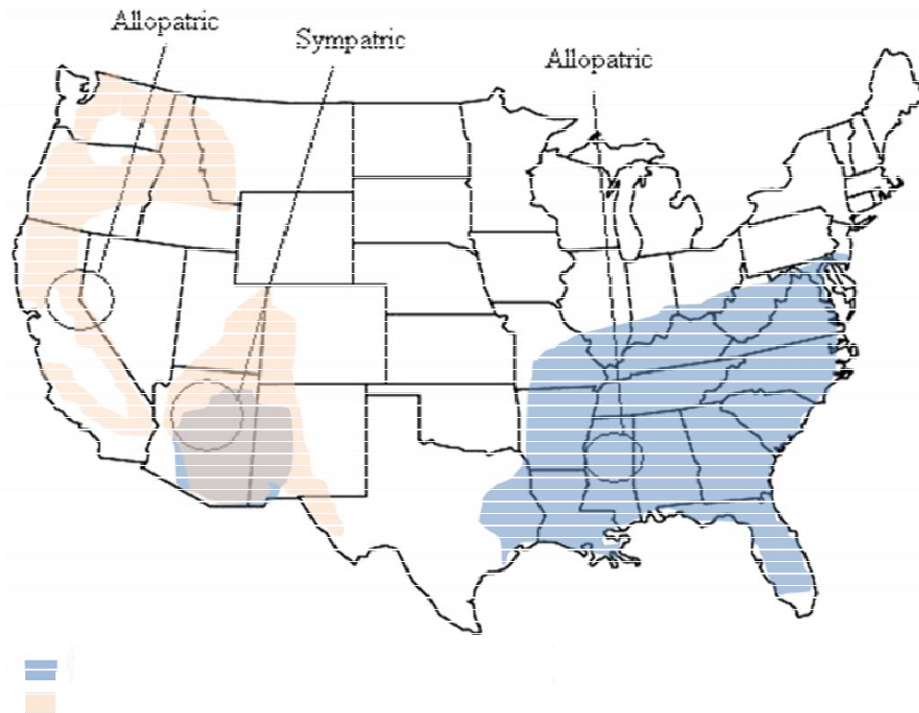
Bark beetles used for fungal isolation were collected from three locations: Coconino National Forest in Arizona, U.S.A., Homochitto Ranger District National Forests in Mississippi, U.S.A., and Plumas National Forest in California, U.S.A. (Figure 1). In Arizona, populations of *D. frontalis* and *D. brevicomis* occur in sympatry and co-colonize ponderosa pine (*Pinus ponderosa* var *brachyptera*). In Mississippi only *D. frontalis* occurs and colonizes multiple pine species. In California only *D. brevicomis* occurs and colonizes *P. ponderosa* var *benthamiana*. There is moderate climatic variability between the three forests in terms of both annual mean precipitation and maximum temperature. On the Coconino National Forest annual precipitation averages 44.7 cm per year and mean summer maximum temperature is 25°C (Hereford, 2007), on the Homochitto National Forest annual precipitation averages 162.5 cm per year and mean summer maximum temperature is 30°C (Southern Regional Climate Center), and on the Plumas National Forest annual precipitation averages 38.8 cm per year and mean summer maximum temperature is 29.6°C (Western Regional Climate Center).

To obtain mycangial fungi, live beetles were trapped in the field using Lindgren funnel traps baited with pine beetle pheromone lures containing frontalin, *exo-brevicomin*, and *-pinene* (Synergy Semiochemicals Corp, Lot No. WPP10416). Beetles were placed individually into clear, size 0 gelatin capsules (Torpac, Lot No. 1100049271), and stored in the lab in dark environmental chambers at 5°C until used for the isolation of fungi. All insect specimens used for microbial isolations mentioned in this study were collected between May 18, 2007 and July 18, 2007. Healthy female beetles were dissected and the thorax removed. Each thorax was surface sterilized using HgCl<sub>2</sub> and de-ionized water described by (Kopper et al., 2004) and then split dorsoventrally and placed in 2% malt extract media (Malt extract – MP Biomedicals LLC, Lot No. 6753J; Agar – BioServ, Lot No. 1740.01). The pH of the media was 4.7 ± 0.2 according to manufacturer specifications. Malt extract media (2%) and 95 × 15 mm Petri dishes (Fisherbrand) were used for all isolations and assays. Dishes containing isolates were sealed using Parafilm and incubated in dark environmental chambers at 15°C until used in assays.

### Fungal identification

Fungal colonies were determined to be one of two fungal genera, *Entomocorticium* and *Ceratocystiopsis*, and were putatively identified based on microscopic observations of hyphal morphology and degree of melanization (Klepzig et al., 2004). Twenty strains of mutualist mycangial fungi from *D. brevicomis* and *D. frontalis* in sympatry (9 *D. frontalis* and 11 *D. brevicomis*), and thirteen fungal strains from allopatric beetle populations (6 from *D. frontalis* in Mississippi and 7 from *D. brevicomis* in California) were isolated. Fungi from *D. brevicomis* and *D. frontalis* are *Ceratocystiopsis brevicomi* and *Entomocorticium* sp. B, and *Ceratocystiopsis ranaculosus* (J.R. Bridges and T.J. Perry) Hausner and *Entomocorticium* sp. A, respectively (Hsiau and Harrington, 1997).

The identity of fungal strains were confirmed by sequencing of internal transcribed spacer (ITS) regions 1 and 2 between the ribosomal RNA genes 18S and 28S. DNA was extracted using a Qiagen DNeasy plant kit (Valencia, CA) with the modified protocol



**Figure 1.** The locations of beetle populations where fungal isolates were collected. *D. brevicomis* and *D. frontalis* occur in sympatry in northern Arizona (Coconino National Forest), *D. brevicomis* in allopatry in California (Plumas National Forest), and *D. frontalis* in allopatry in Mississippi (Homochitto National Forest).

for yeasts, which includes a sorbitol buffer and lyticase enzyme to digest cell walls. The author used primers 5.8SF (5'-CGCTGCGTTCTTCATCG-3') and 5.8SR (5'-TCGATGAAGAACGCAGCG-3') from White et al. (1990) and paired them with newly developed primers ITS-18S (5'-CTTSAACGAGGAATNCCTAGTA-3') and ITS-28S (CATWCCCAAACWACYCGACTC) for ITS regions 1 and 2, respectively (Cindy Liu et al. unpublished manuscript).

The authors used the following parameters for a touchdown PCR: hot start 95°C for 4 min; then 20 cycles at 95°C for 30 s, 60°C for 1 min decreasing 0.5°C each subsequent cycle, 72°C for 1 min; 12 cycles at 95°C for 30 s, 45°C for 30 s, 72°C for 30 s; finishing with 72°C for 7 min. PCR reagents were used in the following final concentrations: Invitrogen PCR buffer 1 x (Carlsbad, CA), primers 0.2 uM each, MgCl<sub>2</sub> 2.5 mM, dNTPs 0.8 uM, and Invitrogen Platinum taq polymerase 1.4 U. PCR amplicons were cleaned up using ExoSAP-IT (USB, Cleveland, OH), cycle sequenced with ABI PRISM BigDye Terminator 3.1 (Applied Biosystems, Foster City, CA), and run on an ABI 3130 x l Genetic Analyzer. Sequences for both reads were edited and compiled in Sequencher 4.9 (Gene Codes, Ann Arbor, MI) and BLASTed against all GenBank accessions. Identifications were based on the highest identity value (complete match) and read length. Representative voucher specimens of fungi were preserved in 80% glycerol/20% malt extract broth (MEB; Difco; Lot No. 7306921) and placed in storage freezers held at -80°C in the Microbial Genetics and Genomics Center in Flagstaff, Arizona, U.S.A.

### Primary resource acquisition by mycangial fungi

Radial growth is a primary mode of resource acquisition for fila-

mentous fungi, and primary resource acquisition was defined as the rate at which fungal colonies occupied media area in the absence of competitors. All fungal strains were incubated in dark environmental chambers at six temperatures of 5, 10, 15, 20, 25 and 28°C. Fungi were transferred from original isolates to sterile 2% malt extract agar by extracting a 1 x 1 mm section of growth media from hyphal tips of isolations during the linear growth phase using a flame-sterilized spatula. Hyphal growth was traced every 48 h beginning at day zero (initial transfer of colony) for 15 d. The growth rate for each fungal colony at each temperature was determined by dividing the distance between tracings by the number of days of growth. This study was replicated twice for each strain and growth rate was quantified in mm growth/day to  $1 \times 10^{-1}$  mm.

### Competitive resource acquisition by mycangial fungi

Combative interaction between organisms is a secondary means of resource acquisition when limited resources are occupied by competitors (Tilman, 1982). The authors divided competitive resource acquisition into two parts: (1) Resource acquisition/capture: the mean frequency with which a fungal isolate colonized media resources occupied by a competing fungal isolate, and (2) Resource defense: the mean frequency with which a fungal strain resisted colonization by a competing fungal colony. Competitive interactions between mycangial fungi were tested using a pairwise approach. All fungi were paired in Petri dishes by placing 1 x 1 mm media sections containing fungal hyphae at opposing ends of the dish, and fungal strains were transferred from original isolate colonies as described above. Each isolate was also tested against itself. This full factorial design (33 x 33) was replicated twice (n = 2178 assays).

Variation in the growth patterns of these fungi on 2% malt extract

**Table 1.** Fungal growth rates (mm/day<sup>-1</sup>) at multiple temperatures and passive / competitive resource acquisition patterns by fungal species and beetle host isolated from sympatric beetle populations. ANOVA results also shown. Bold values indicate significance differences in means at = 0.05.

| Variable         | Beetle species       |                     | Fungal genera           |      |       |                        |                 |       |      |       |
|------------------|----------------------|---------------------|-------------------------|------|-------|------------------------|-----------------|-------|------|-------|
|                  | <i>D. brevicomis</i> | <i>D. frontalis</i> | <i>Ceratocystiopsis</i> |      |       | <i>Entomocorticium</i> |                 |       |      |       |
|                  | Mean ± SE            | Mean ± SE           | F                       | df   | P     | Mean ± SE              | Mean ± SE       | F     | df   | P     |
| 5°C              | 0.091 ± 0.033        | 0.040 ± 0.029       | 1.312                   | 1.16 | 0.268 | 0.074 ± 0.027          | 0.057 ± 0.036   | 0.141 | 1.16 | 0.711 |
| 10°C             | 0.425 ± 0.117        | 0.368 ± 0.103       | 0.133                   | 1.16 | 0.720 | 0.446 ± 0.094          | 0.347 ± 0.127   | 0.372 | 1.16 | 0.550 |
| 15°C             | 0.704 ± 0.150        | 0.665 ± 0.131       | 0.040                   | 1.16 | 0.843 | 0.592 ± 0.121          | 0.777 ± 0.162   | 0.808 | 1.16 | 0.381 |
| 20°C             | 1.086 ± 0.254        | 1.361 ± 0.222       | 0.660                   | 1.16 | 0.428 | 1.016 ± 0.205          | 1.432 ± 0.276   | 1.410 | 1.16 | 0.252 |
| 25°C             | 1.518 ± 0.299        | 1.999 ± 0.262       | 1.452                   | 1.16 | 0.245 | 1.584 ± 0.242          | 1.933 ± 0.325   | 0.715 | 1.16 | 0.409 |
| 28°C             | 1.250 ± 0.228        | 0.95 ± 0.152        | 1.193                   | 1.16 | 0.306 | 1.320 ± 0.216 a        | 0.880 ± 0.125 b | 3.100 | 1.16 | 0.116 |
| Resource defense | 0.488 ± 0.047        | 0.497 ± 0.041       | 0.016                   | 1.16 | 0.900 | 0.594 ± 0.038 a        | 0.391 ± 0.051 b | 9.707 | 1.16 | 0.006 |
| Resource capture | 0.386 ± 0.076        | 0.425 ± 0.067       | 0.141                   | 1.16 | 0.712 | 0.445 ± 0.061          | 0.366 ± 0.183   | 0.571 | 1.16 | 0.460 |

\*Letters indicate differences in means (Tukey's HSD test) by row.

media is consistent with that found in tree phloem (Rayner and Webber, 1984; Klepzig and Wilkens, 1997; Hofstetter et al., 2005). Following establishment of strains on dishes (1 - 2 d); hyphal growth was traced every 48 h for 30 d. Assays were done at 25°C in the dark. The outcomes of paired competition assays are reported in terms of the mean frequency of resource acquisition and the mean frequency of resource defense by each fungal strain. These two observational metrics yielded four basic outcome categories for each colony in each pairing: (1) Fungal isolate A grew over media colonized by its paired competitor fungal isolate B; (2) Media colonized by fungal isolate A was grown over by its paired competitor fungal isolate B; (3) Both fungal isolates A and B successfully grew into others colonized area, or (4) Both fungal isolates A and B resisted overgrowth or formed a partition (Tuininga, 2005). Thus, each isolate in each trial received both a resource capture score and a defense score. Scoring for every pairing was verified microscopically (10 - 100 x magnification) by examining hyphal interactions.

The outcomes (mean frequency of resource capture and resource defense) of paired competition assays were converted "a posteriori" to binary values (0 = failure to defend/capture; 1 = successful defense/capture) for each isolate and averaged over all assays to yield an index of each fungal isolates' competitive performance on a continuous scale. Thus, each isolate received a relative frequency score (ranging from 0 - 1) that described the proportion of competitive resource acquisitions and defensive responses. For example, an isolate with a resource defense score of 0.87 indicates that the fungi successfully resisted colonization in 87% of competition trials.

### Statistical analyses

All statistics were computed using JMP 7.0 software (SAS Institute). Statistical tests were prefaced by checking statistical assumptions. Assumptions of normality were verified using a Shapiro Wilk Test, and no transformations were required. In comparisons among fungi in sympatry (n = 20 sympatric fungal isolates), a two-way ANOVA was performed to analyze the fixed effects of beetle species, fungal species, and beetle species x fungal species interaction on response variables of radial growth rate, mean resource capture and mean resource defense. The beetle species x fungal species interaction effect did not contribute significant variation to the statistical model, so the interaction effect is not reported in the ANOVA summary for ease of display.

In comparisons between isolates across beetle populations (n = 33 fungal strains [20 sympatric/13 allopatric]), a one-way ANOVA was performed to analyze the fixed effect of sympatry or allopatry on response variables of radial growth rate, mean resource acquisition and mean resource defense. For this analysis sympatric isolates were considered as a single (Arizonan) population. The effects of temperature on fungal growth were analyzed as a fixed effect nested by location and differences between means were tested using contrasts. Statistical significance was established at = 0.05 for statistical tests and ANOVA models for growth and competition assays were analyzed using F-tests to establish the significance of effects. Where differences in mean growth and competitive responses were detected in ANOVA models, directionality was established using Tukey's HSD Test.

## RESULTS

### Primary resource acquisition by mycangial fungi

**Sympatric fungi:** Growth rates of fungi from Arizona did not vary by beetle species, fungal species, or beetle species x fungal species interaction at 5, 10°C, 15, 20, 25 or 28°C (Table 1, Figure 2). Thus, fungi from sympatric beetle populations behaved statistically identically in terms of growth rates across temperatures by both fungal species and beetle species. However, fungal growth rates did consistently increase as temperature increased then declined once ambient temperature surpassed 28°C. *Entomocorticium* species exhibited greater variability in growth rates across temperatures than *Ceratocystiopsis* species.

**Sympatric and allopatric fungi:** Growth rates did not vary among the three populations at 5 or 10°C (Table 2, Figure 2). Primary resource acquisition from the sympatric fungi was significantly higher than the rate of primary resource acquisition by fungi from *D. brevicomis* in California at 15 and 20°C, and fungi from *D. frontalis* in Mississippi were intermediate. There was no difference in primary resource acquisition by population at 25°C, but at 28°C primary resource acquisition by sympatric isolates

**Table 2.** Fungal growth rates (mm/day<sup>-1</sup>) at multiple temperatures and passive / competitive resource acquisition patterns by fungal species and beetle host isolated from sympatric and allopatric beetle populations. ANOVA results also shown. Bold values indicate significance differences in means at = 0.05.

| Variable         | Population   |                                     |                                    | F      | df   | P      |
|------------------|--|-------------------------------------|------------------------------------|--------|------|--------|
|                  | Sympatric ( <i>D. brevicomis</i> and <i>D. frontalis</i> ) | Allopatric ( <i>D. brevicomis</i> ) | Allopatric ( <i>D. frontalis</i> ) |        |      |        |
| 5°C              | 0.064 ± 0.034  | 0.034 ± 0.054                       | 0.187 ± 0.060                      | 2.199  | 2.30 | 0.137  |
| 10°C             | 0.408 ± 0.065  | 0.237 ± 0.079                       | 0.356 ± 0.086                      | 1.367  | 2.30 | 0.277  |
| 15°C             | 0.799 ± 0.084 a  | 0.428 ± 0.100 b                     | 0.588 ± 0.108 ab                   | 4.093  | 2.30 | 0.032  |
| 20°C             | 1.341 ± 0.180 a  | 0.577 ± 0.216 b                     | 1.136 ± 0.233 ab                   | 3.752  | 2.30 | 0.041  |
| 25°C             | 2.016 ± 0.174 a  | 0.841 ± 0.259 b                     | 1.810 ± 0.280 a                    | 6.386  | 2.30 | 0.007  |
| 28°C             | 1.100 ± 0.201 b  | 2.362 ± 0.241 a                     | 2.987 ± 0.260 a                    | 18.233 | 2.30 | <0.001 |
| Resource defense | 0.800 ± 0.031 a  | 0.707 ± 0.038 b                     | 0.616 ± 0.041 b                    | 3.593  | 2.30 | 0.046  |
| Resource capture | 0.496 ± 0.078 a  | 0.185 ± 0.101 b                     | 0.210 ± 0.109 b                    | 3.533  | 2.30 | 0.050  |

\*Letters indicate differences in means (Tukey's HSD test) by row.

was significantly lower than for allopatric isolates (Figure 2). Growth rates consistently increased with temperature for all fungal isolates until 25°C, where sympatric isolates showed a substantial decline but allopatric fungi achieved optimal growth.

### Competitive resource acquisition by mycangial fungi

Sympatric fungi: Patterns of competitive resource acquisition varied significantly with fungal species but were not variable with beetle species (Table 1). Specifically, *Ceratocystiopsis* species had higher mean frequencies of resource defense than *Entomocorticium* species (Table 1). Thus, neither beetle species was associated with a consistently more competitive fungal symbiont. Sympatric and allopatric fungi; Competitive resource acquisition by fungal isolates varied significantly with sympatry and allopatry of source beetle populations (Table 2). Isolates from the

sympatric beetle populations exhibited significantly higher mean frequencies of competitive resource acquisition and resource defense of growth media than fungi isolated from either allopatric beetle population (Table 2). Fungi from opposing allopatric populations were not significantly different from each other.

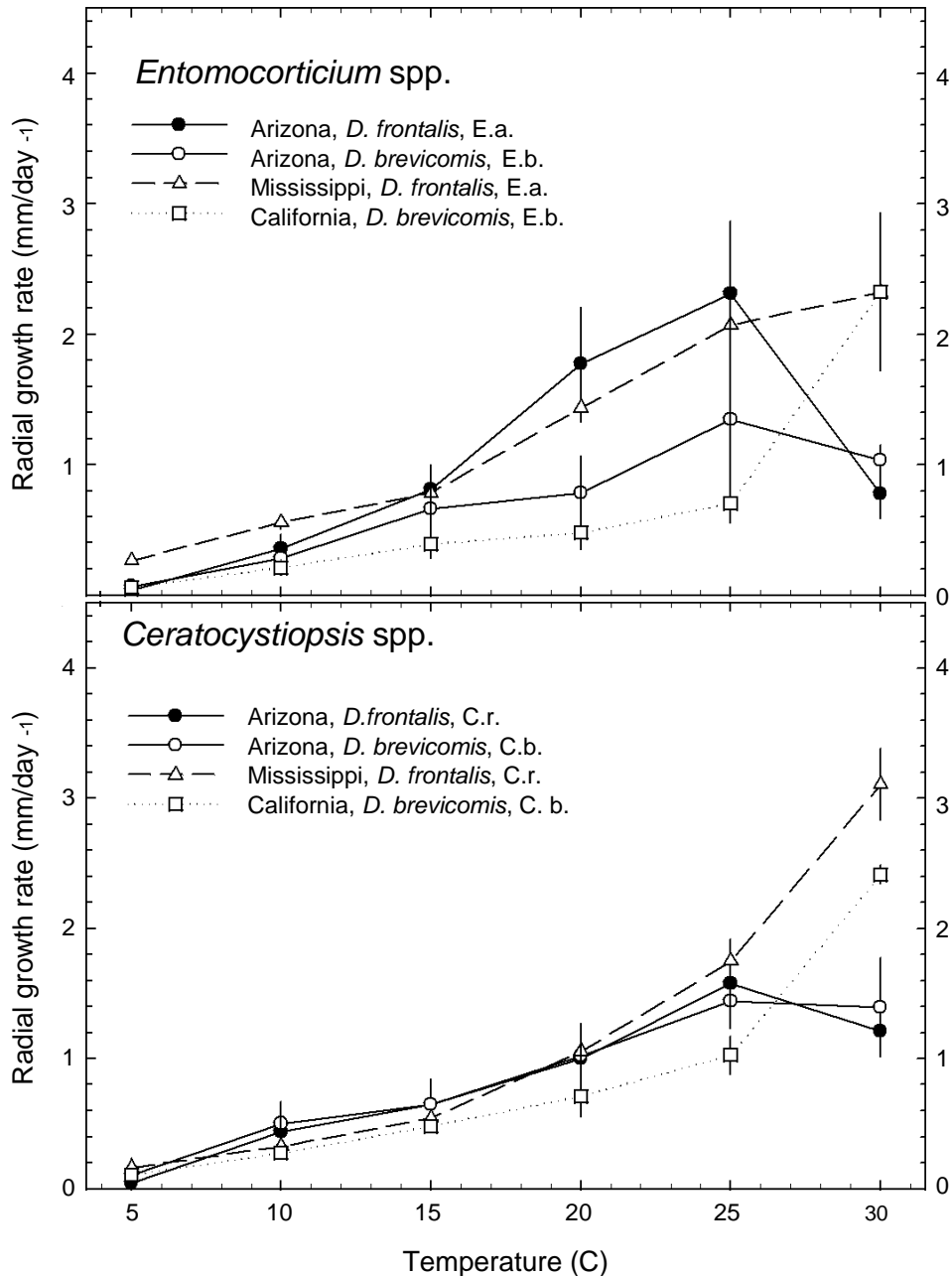
## DISCUSSION

### Primary resource acquisition

The radial growth rates of fungi were found to vary by population at multiple temperatures (Table 2). This is in agreement with the findings of Six and Bentz (2007), which showed that ambient temperature was a mediator of fungal abundances, and that this variation was related to both site and seasonality in a *Dendroctonus ponderosae* system. Here, they show that the growth rates of mycangial fungi vary across beetle populations, which in the current study are separated by large

geographic regions. However, growth rates did not vary among fungal isolates for fungal species or beetle species within sympatric populations. The studies did not sample fungi from multiple sites within sympatric populations, so they might have detected greater variation in growth rates by assessing multiple sites within each region.

In contrast to the present study, Hofstetter et al. (2007) showed that *Entomocorticium* sp. A, exhibited optimal growth at a lower ambient temperature than *C. ranaculosus* in a *D. frontalis* system in Mississippi. In the present study, no differences in response to temperature were detected for growth by fungal species from sympatric beetle populations in Arizona or an allopatric beetle population in California (Figure 2). However, the authors did support their findings that *Entomocorticium* sp. A, and *C. ranaculosus* had different optimal growth rates in a Mississippi population of *D. frontalis* (Figure 2). One explanation for this pattern is overall variability in daily temperature: daily temperature range is greater in Arizona and California than in Mississippi



**Figure 2.** Growth rates of *Ceratocystiopsis* spp. and *Entomocorticium* spp. isolated from sympatric bark beetle populations in Arizona, an allopatric population of *D. brevicomis* in California, and an allopatric population of *D. frontalis* in Mississippi. Sympatric populations were pooled in this figure since there were no significant differences between radial growth rates of sympatric fungi. Error bars represent one standard error.

(Hereford, 2007; Southern Regional Climate Center, 2008; Western Regional Climate Center, 2008). Thus, optimal growth rate and species abundances of mycangial fungi may be influenced by daily temperature range, in addition to regional and seasonally mediated temperature differences. The rates of primary resource acquisition by sympatric fungi corresponded to the average maximum summer temperature in the region. In northern Arizona, climate records show that maximum

summer (June-September) temperatures average 25°C (Hereford, 2007), which is the range where the greatest average growth rates were observed for sympatric fungi (Figure 2). In the experiments, an increase in ambient temperature of only 3°C above the average summer maximum correlated with a dramatic decrease in growth rates of the sympatric fungi. In contrast, mycangial isolates from allopatric populations showed no apparent decrease in growth rates as ambient temperatures

increased, and isolates from both populations grew optimally at 28°C. Unfortunately, no inferences can be made about primary resource acquisition by fungi beyond ambient temperatures of 28°C. However, previous work shows that the growth rates of mycangial fungi (both *Entomocorticium* and *Ceratocystiopsis*) isolated from *D. frontalis* in Mississippi declines once ambient temperatures exceeds 28°C (Hofstetter et al., 2007). If it is true that fungal growth rates correspond to regionally defined average maximum temperatures during summer months, then they would predict that isolates from allopatric *D. brevicomis* will grow optimally at 29.6°C, and isolates from allopatric *D. frontalis* will grow optimally at 30°C. Future studies with these organisms could benefit from assaying growth rates of mycangial fungi from both within and between beetle populations across a broader temperature range and at smaller intervals, since isolates appear to be highly sensitive to relatively small incremental variations in temperature.

### Competitive resource acquisition

Fungi isolated from the sympatric beetle populations exhibited significantly greater frequencies of competitive acquisition and defense of media in a resource-limited environment. In sympatric beetle populations, *Ceratocystiopsis* species defended media resources from colonization by opposing isolates with significantly higher frequency than *Entomocorticium* species (Table 1). Data regarding competitive resource acquisition and resource defense by allopatric fungi were strongly asymmetric: in almost, no case (< 5%) was a fungal isolate from an allopatric beetle population able to colonize media occupied by a fungal isolate from a sympatric beetle population. Thus, competitive resource acquisition by allopatric isolates occurred almost exclusively in pairings with other allopatric isolates. The opposite was not true for sympatric fungi, which were able to competitively acquire media resources colonized by isolates from all beetle populations. However, all competition assays were performed at 25°C, where sympatric fungi exhibited their optimal growth. Thus, it is possible that the observed differences in competitive performance between sympatric and allopatric isolates were due to localized adaptations to temperature or the seasonality of collection (Six and Bentz, 2007). However, optimal growth rate of mycangial fungi from *D. frontalis* collected in Mississippi and Alabama is reported between 25 - 28 C (Klepzig et al., 2001; Hofstetter et al., 2007) . The authors suggest that future studies related to competition between multiple mycangial species focus on testing interactions across a broader range of temperatures. In nature, many factors may contribute to the outcomes of competitive interactions between multiple fungi. For example, Licyayo et al. (2007) found that ammonia concentrations and pH had strong effects on interspecific

interactions between fungal species. Similarly, melanin and other pigments have been shown to strongly mediate competition between fungal species (Yuen et al., 1999; Klepzig, 2006). In a *D. frontalis* system, water availability was determined to play an important role in fungal competition (Klepzig et al., 2004). The studies only account for differences in competitive ability among fungi between regions of sympatry and allopatry, and by beetle species and fungal species within sympatric populations. However, the present study represents a first assessment of combative interactions between the mutualists of two or more bark beetle species, and suggests that fungi are likely to adapt to a competitive environment when multiple mycophagous beetle species inhabit a single plant host. Future studies of competitive interactions could benefit by testing interspecific fungal interactions across a gradient of host plant *Dendroctonus* beetles are frequently exposed to a terpenoid-saturated environment during the colonization of host tissues, and exposure to terpenoid compounds strongly impacts the growth performance of beetle – associated fungi (Paine and Hanlon, 1994; Hofstetter et al., 2005).

In conclusion, the mycangial fungi associated with *D. brevicomis* and *D. frontalis* were variable with respect to primary resource acquisition and competitive interactions. Growth rates of fungi did not vary by beetle species or fungal species when beetle populations were sympatric, however fungal growth rates did show substantial variation across regions. In sympatric populations, *Ceratocystiopsis* species were more likely to resist colonization by a competitor than *Entomocorticium* species. Mycangial isolates from sympatric beetle populations were more likely to competitively acquire resources. Interactions between sympatric isolates and allopatric isolates were asymmetric: sympatric isolates were better competitors and frequently colonized media resources inhabited by an allopatric isolate. Interestingly, competition also appeared to be symmetric among allopatric isolates.

These data reported here support the hypothesis that interactions between mycangial fungi of multiple *Dendroctonus* species are antagonistic (-, -), since fungi that occurred in sympatry were stronger competitors. Furthermore, these findings may be extendable to other systems where multiple insects with fungal associates colonize the same plant host and insect -fungal associations have been increasingly recognized as a central theme in arthropod ecology (Blackwell and Vega, 2005). The importance of these interactions for beetle larval performance and fungal-beetle relationships are still unknown and further experiments are needed to determine how competition between multiple fungal associates of *Dendroctonus* species affect beetle fitness.

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