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Leptographium pyrinum is a Mycangial Fungus of *Dendroctonus adjunctus*

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Leptographium pyrinum is a mycangial fungus of *Dendroctonus adjunctus*

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Abstract: Several species of *Dendroctonus* (Coleoptera: Scolytidae) have cuticular invaginations, or mycangia, in the integument which are specialized for carrying specific symbiotic fungi. The mycangium of *Dendroctonus adjunctus,* located under a callus that surrounds the prothorax, has been recognized but the mycangial fungus has not yet been identified. Fungi from mycangia of *Dendroctonus adjunctus* were isolated and compared with *Leptographium pyrinum* and *Ophiostoma adjuncti,* two species of fungi known to be present in trees colonized by *Dendroctonus adjunctus.* Fungi isolated from *Dendroctonus adjunctus* mycangia were determined to be morphologically and genetically identical to *Leptographium pyrinum.*

Key Words: Ascomycetes, bark beetles, mycangia, mycangial fungi, *Ophiostoma adjuncti*

INTRODUCTION

The round-headed pine beetle, *Dendroctonus adjunctus* Blandford, is an aggressive bark beetle attacking seven species of *Pinus* L. in southwestern North America and in Central America (Wood, 1982). Adult females possess a mycangium located within a pronotal callus which surrounds the prothorax (Wood, 1963; Francke-Grosmann, 1967). These mycangia are gland-lined structures specialized for carrying specific fungi (Batra, 1963; Francke-Grosmann, 1967; Barras and Perry, 1971; Happ et al., 1971).

The presence of complex structures such as mycangia and the persistence of the associations between insect and fungus suggest that mycangial fungi play an essential role in the fitness and survival of their associated bark beetles. The relationship between the beetles and their mycangial fungi has long been assumed to be mutualistic, however, the benefits gained by each member in these associations remain poorly understood. The fungus benefits in being transported by the beetle to the host tree on which it is dependent for growth and reproduction. The beetle may benefit from the association in various ways. The fungus may reduce detrimental interactions with antagonistic blue-staining fungi (Barras, 1970; Franklin, 1970; Whitney, 1971; Whitney and Cobb, 1972 but see Ross et al., 1992), aid the beetles in overcoming the defenses of the tree (Paine and Stephen, 1987), alter the chemical or moisture composition of the phloem (Nelson, 1934; Reid, 1961; Graham, 1967; Webb and Franklin, 1978; Wagner et al., 1979) or provide nutrients essential for some aspect of reproduction or development (Barras, 1973; Bridges, 1983; Goldhammer et al., 1990).

Four North American species of *Dendroctonus* possess prothoracic mycangia: *D. frontalis* Zimmermann, *D. brevicomis* LeConte, *D. adjunctus,* and *D. approximatus* Hopkins. *Dendroctonus frontalis* and *D. brevicomis* are closely related species and carry both nonbluestaining ophiostomatoid fungi and basidiomycetes of uncertain taxonomic affinity in their mycangia. The mycangial fungi of *D. adjunctus* and its sister species, *D. approximatus,* have not been identified. Barras and Perry (1971), however, isolated a *Verticicladiella* S. Hughes-like (=*Leptographium* Lagerb. and Melin) fungus that appeared similar to *Ceratocystis huntii* (Robins.-Jeff.) [≡*O. huntii* (Robins.-Jeff.) deHoog and Scheffer] from the mycangium of *D. adjunctus.* Like *O. huntii,* the mycangial fungus had conidia that possessed well developed abscission scars but differed in having a broader conidial size range and the conidia were more obovate in shape. Later, Davidson (1978) described *Leptographium pyrinum* and *Ceratocystis adjuncti* [≡*O. adjuncti* (Davids.) Harrington] from *D. adjunctus* infested pines in New Mexico. The mycangial fungus isolated by Barras and Perry (1971) has not been compared with *L. pyrinum* or *O. adjuncti* (Harrington, 1988).

The objective of this study was to compare fungal isolates obtained from mycangial dissections of *D. adjunctus* with *L. pyrinum* and *O. adjuncti* using morphology and isozymes to determine if either of these fungi commonly associated with *D. adjunctus* are carried in the beetles' mycangia.

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TABLE I. Origin of fungal strains isolated from *Dendroctonus adjunctus*

	Locality	Host tree[a]	Isolate#
L. pyrinum	New Mexico	Unknown	C96[b]
Mycangial fungi	Riggs Flat, Pinaleno Mtns, Arizona	PP	DLS787
	Twilight Crk, Pinaleno Mtns, Arizona	AP	DLS791
	Riggs Flat, Pinaleno Mtns, Arizona	WP	DLS877
	Riggs Flat, Pinaleno Mtns, Arizona	WP	DLS878
	Turkey Flat, Pinaleno Mtns, Arizona	AP	DLS879
	Turkey Flat, Pinaleno Mtns, Arizona	AP	DLS882
	Arcadia Cmp, Pinaleno Mtns, Arizona	CP	DLS883
	Twilight Crk, Pinaleno Mtns, Arizona	AP	DLS890
	Riggs Flat, Pinaleno Mtns, Arizona	PP	DLS903
	Riggs Flat, Pinaleno Mtns, Arizona	WP	DLS904
	Turkey Flat, Pinaleno Mtns, Arizona	AP	DLS911
O. adjuncti	New Mexico	PP	C119[b]

[a] PP = *P. ponderosa*, AP = *P. arizonica*, WP = *P. monticola*, CP = *P. leiophylla*.
[b] Collected by R.W. Davidson. All others collected by Diana L. Six.

MATERIALS AND METHODS

Dendroctonus adjunctus adults were collected from *Pinus ponderosa* Laws., *P. arizonica* Engelm., *P. leiophylla* Schiede et Deppe, and *P. monticola* Dougl. from several locations in the Pinaleno Mountains, Coronado National Forest, Arizona. Live beetles removed from trees were immediately placed individually into plastic microcentrifuge tubes which were then placed into plastic bags and placed on ice until returned to the laboratory. In the laboratory the beetles were held live for up to two wk in a refrigerator (ca. 5 C) until they were dissected.

Female beetles were rinsed with sterile water and dissected by removing the head and abdomen from the thorax. Any remaining internal tissues still attached to the thorax were scraped away. The thorax was surface sterilized using a series of washes (1 min sterile water, 4 min improved White's solution, 1 min sterile water, 1 min sterile water) (Barras, 1972). The mycangia were then trimmed from the thorax using scissors sterilized by soaking in 95% ethanol, clipped into several pieces, and the pieces placed onto *Ophiostoma* selective medium (2% malt agar containing 100 ppm cycloheximide and 10 ppm streptomycin) (Harrington, 1981). Portions of mycangia of several beetles were also placed onto malt agar. Small portions of mycelium growing out from the pieces of mycangia were then sub-cultured onto malt agar.

One culture each of *L. pyrinum* and *O. adjuncti* were obtained from Dr. Thomas C. Harrington, Iowa State University, Ames, for comparison with strains obtained from mycangial dissections. Both cultures are apparently from the holotype of the respective species. The origins of these and other strains used in this study are listed in TABLE I. All strains have been deposited in the culture collection of the senior author. The strains DLS791, DLS878, DLS879, and DLS883 have also been deposited in the culture collection of T. C. Harrington, Iowa State University, Ames.

Several morphological characteristics of the anamorphs produced by each strain were compared from cultures grown on malt agar in darkness at room temperature (ca. 22 C). Conidial dimensions were compared with paired t-tests using StatView statistical software (Abacus Concepts, 1991).

The fungi were compared genetically using horizontal starch gel electrophoresis and isozymes. Mycelium for enzyme extraction was obtained by placing agar plugs of each isolate into 30 mL liquid medium (20 mg malt extract, 1 mg yeast extract/mL) in 125-mL Erlenmeyer flasks for 14 d at 21 C (Zambino and Harrington, 1992). Enzymes were extracted by grinding the fungi in liquid nitrogen and then in extraction buffer (Zambino and Harrington, 1989). The liquid extract was absorbed onto 4 mm #1 Whatman filter paper wicks and loaded onto 10% horizontal starch gels (Sigma starch) along with reference wicks. Following electrophoresis, gels were sliced horizontally and stained for enzyme activity. Buffer systems and staining procedure references are listed in TABLE II. Nine enzymes exhibiting twelve putative genetic loci that resolved well were selected (TABLE II). Different electromorphs were assumed to be the result of different alleles.

RESULTS

Twenty-seven strains of fungi were isolated from the mycangia of 38 adult female *D. adjunctus* collected from four species of host tree. Fungi were successfully isolated on both malt agar (5 isolates) and *Ophiosto-*

TABLE II. Enzymes, buffers, and staining procedures used in starch gel electrophoresis of *L. pyrinum, O. adjuncti,* and the *D. adjunctus* mycangial fungus

Enzyme (EC number)[a]	Abbreviation[b]	Buffer systems[c]	Stain references[d]
Aconitase (4.2.1.3)	ACO1	A[1]	1
Diaphorase (1.6.4.3)	DIA2	A	2
Fumarase (4.2.1.2)	FUM	A	2
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6PD	A	1
Glucosephosphate isomerase (5.3.1.9)	GPI	D[2]	1
Isocitrate dehydrogenase (1.1.1.42)	IDH	E[3]	1
Leucine aminopeptidase (3.4.11.1)	LAP1	M[4]	2
Malate dehydrogenase (1.1.1.37)	MDH1,2	D, E	2
Menadione reductase (1.6.99.2)	MNR2,3	A	1

[a] Code of the Nomenclature Committee of the International Union of Biochemistry (1984).

[b] Multiple forms of enzymes are designated in order of decreasing anodal migration.

[c] 1 = Marty et al. (1984). 2 = Conkle et al. (1982). 3 = Conkle unpubl. in Zambino and Harrington (1989). 4 = Micales et al. (1986).

[d] Conkle et al. (1982). 2 = Marty et al. (1984).

ma selective agar (27 isolates). When fungi were isolated from a single beetle on both media, only isolates from the selective medium were used. Twenty-six of the strains were morphologically similar to *L. pyrinum.* The mean length and width (± SE) of conidia from the reference strain of *L. pyrinum* were 7.63±2.60 and 4.40±0.26, respectively. The mean length and width (±SE) of the mycangial fungus strains were 7.64±2.46 and 4.72± 0.37, respectively. Comparisons of conidial dimensions for these mycangial fungus strains and the *L. pyrinum* reference strain found no significant differences (for length of conidia df=24, t=−0.61, P=0.56 and for width of conidia df=24, t=−0.99, P=0.17). Anamorph and cultural characteristics for *L. pyrinum* and for the mycangial fungus strains are given in TABLE III along with descriptions given by Barras and Perry (1971) of the fungus they isolated from *D. adjunctus* mycangia and the formal description of *L. pyrinum* (Davidson, 1978). The range of size of conidia fit very closely with the range of conidial size found by Barras and Perry (1971) for the fungus they isolated from mycangia of *D. adjunctus.* Davidson (1978) gives a range of larger conidia, but other characteristics described by Davidson for *L. pyrinum* fit very closely with those of the mycangial strains, including the common occurrence of swollen cells in the conidiophores. Cultures of the mycangial fungi were very similar in appearance to the reference strain of *L. pyrinum,* and growth rates were similar as well. One strain isolated from *D. adjunctus* mycangia was morphologically similar to the reference strain of *O. adjuncti.*

Ten strains isolated from mycangia that were morphologically similar to the reference strain of *L. pyr-*

TABLE III. Characteristics of *Leptographium pyrinum* and the morphologically and culturally similar mycangial fungus strains

	Strains used in this study		Description of Barras and Perry (1971)	Formal description Davidson (1978)
	L. pyrinum	Mycangial fungus		
Colony morphology at 22C	Brown becoming black, appressed, submersed, masses of white sticky conidia	Same as *L. pyrinum*	No description given	Brown, becoming black. White conidial masses.
Growth rate	Fills 90 mm plate in 8 d at 22 C	Same as *L. pyrinum*	No description given	Fills 90 mm plate in 10 d (no temp. given)
Conidia: Shape	Pyriform, obovate, abscission scar	Same as *L. pyrinum*	Obovate, abscission scar	Pear-shaped, broadly ovoid
Size (μm)	10.0–3.75 × 5.0–3.0	11.3–3.8 × 7.5–2.5	13.2–3.6 × 8.4–1.2	15–8 × 10–7
Color	Hyaline	Same as *L. pyrinum*	Not given	Not given

inum and the single strain that was morphologically similar to the reference strain of *O. adjuncti* were used in horizontal starch gel electrophoresis. The mycangial strains that were morphologically similar to *L. pyrinum* shared alleles at all loci with the reference *L. pyrinum* strain. All loci in *L. pyrinum* were monomorphic except ACO which had two alleles; Strains from Riggs Flat shared one allele with the reference strain of *L. pyrinum*. Mycangial fungus isolates collected from other areas in the Pinaleno Mountains as well as Riggs Flat exhibited the second ACO allele.

The single strain isolated from *D. adjunctus* that was morphologically similar to the culture of *O. adjuncti* had identical alleles to the *O. adjuncti* reference strain for ten of eleven loci. These two strains shared alleles with *L. pyrinum* at only four loci; FUM, MDH2, DIA2, and LAP1.

DIA1 and MNR1 did not resolve well so were not used. LAP2 was monomorphic and resolved for *O. adjuncti* but not *L. pyrinum*. MNR3 was monomorphic and resolved well for *L. pyrinum* but not for *O. adjuncti*.

DISCUSSION

Mycangia have arisen independently in *Dendroctonus* at least twice. Four closely related *Dendroctonus* species (Bentz and Stock, 1986) have morphologically similar pronotal mycangia. *Dendroctonus brevicomis* and *D. frontalis* are sister species and difficult to separate morphologically. *Dendroctonus adjunctus* and *D. approximatus* are also sister species and morphologically similar. Pronotal mycangia are lined with glandular cells whose secretions may select for particular fungal associates and may also promote fungal growth within the mycangium (Franke-Grosmann, 1967; Happ et al., 1971; Barras and Perry, 1972; Whitney and Cobb, 1972; Paine and Birch, 1983). A second mycangial type is exhibited by *D. ponderosae* Hopkins and *D. jeffreyi* Hopkins which possess maxillary mycangia functional in both male and female beetles (Whitney and Farris, 1970; T.D.P., personal observation). It is unknown whether these mycangia possess secretory cells.

The mycangial ascomycetes carried in the mycangia of *D. brevicomis* and *D. frontalis* are species of *Ceratocystiopsis* (Harrington and Zambino, 1990; Harrington, 1993) and do not stain wood (Davidson, 1966; Whitney and Cobb, 1972; Paine and Birch, 1983; Harrington and Zambino, 1990). It might be expected that the fungi carried by the two groups of sister species with similar mycangial types would be similar. However, *D. adjunctus* was found to carry *L. pyrinum,* and possibly *O. adjuncti,* both of which are capable of blue-staining the wood they infect. Due to the use of a selective cycloheximide-containing medium in this study, other fungi carried in the mycangia of *D. adjunctus* may have been overlooked. *Ceratocystiopsis* spp. grow slowly or not at all on cycloheximide-containing agar and basidiomycetes are completely inhibited (T. C. Harrington, personal communication). Therefore, future isolations should utilize less selective media in order to determine the full complement of mycangial fungi carried by this beetle.

Blue-staining fungi are not unknown as mycangial associates in *Dendroctonus*. The species possessing the maxillary type of mycangium carry yeasts and blue-staining fungi in their mycangia. *Dendroctonus ponderosae* carries *O. clavigerum* (Robins.-Jeff. & Davids.) Harrington and *O. montium* (Rumbold) von Arx, while its sibling species, *D. jeffreyi* Hopkins, carries an undescribed fungus similar or identical to *O. clavigerum,* all of which are blue-staining fungi (Whitney and Farris, 1970; D.L.S. personal observation). It is interesting to note that *O. clavigerum* and *L. pyrinum* are apparently very closely related. Zambino and Harrington (1992) were unable to distinguish between the two fungi using isozymes and suggested that they may be morphological variants of the same species.

The role of mycangial fungi in these associations is unclear. It has been hypothesized that the beetles require the fungi to kill the tree since the tree must be killed in order for the beetle to successfully colonize it and produce brood. If this is true, a very specific association between a beetle species and a single highly virulent fungus might be expected. However, *Dendroctonus* beetles possessing mycangia usually carry two or more fungi, some of which are not pathogenic or are only weakly virulent in artificial inoculations (Strobel and Sugawara, 1986; Owen et al., 1987; Yamaoka et al., 1990; Ross et al., 1992). The sister species of *D. adjunctus, D. approximatus,* is a secondary invader in trees killed by other beetles, disease, or environmental factors (Wood, 1982) and often develops within individual trees simultaneously with *D. adjunctus*. It carries an unidentified blue-staining fungus in its mycangium (D.L.S., unpublished data). If mycangial fungi function primarily to kill the host tree, the maintenance of this association in a non-aggressive beetle species is surprising. However, if the primary role of the fungi is to provide nutritional benefits, the continuance of such an association might be expected. Also, if the fungi benefit the beetles by providing nutrients, then such associations conceivably could be less specific with perhaps a number of fungi being able to provide an equivalent benefit.

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