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Ultrastructure of the mycangium of the southern pine beetle, *Dendroctonus frontalis* (Coleoptera: Curculionidae, Scolytinae): complex morphology for complex interactions

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Abstract

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The southern pine beetle (SPB) (*Dendroctonus frontalis* Zimmermann) is the most economically important pest of southern pine forests. Beetles carry fungal cells within specialised cuticular structures, called mycangia. Little is known about the mycangia ultrastructure or function. We used cryo-fracturing and scanning electron microscopy to examine the ultrastructural features of SPB mycangia and surrounding tissues. Mycangia, one on each side of anterior portion of the prothorax, are terminated on the dorsal side at a 'mycangial bridge'. This sclerotised mycangial bridge does not appear to provide a passage between the two mycangia, suggesting that each mycangium functions independently. Mycangia are surrounded by abundant tracheoles connecting the structures to the outside via openings within the prothorax. Previously unknown pits overlying the mycangial gland cells were also observed in both the inner wall and anterior fold of prothorax. We hypothesise that these openings and pits may play roles in determining which fungi enter, and grow within, the mycangium.

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Introduction

The southern pine beetle (SPB), *Dendroctonus frontalis* Zimmermann (Coleoptera: Curculionidae: Scolytinae), is the most significant pest of southern pine forests. The beetle exhibits different host colonisation behaviours based on the different population densities. At low population levels, SPB breeds in stressed or dying trees of several species of pines. Natural defence mechanisms of healthy trees usually repel or kill any assailants (Smith 1966; Hodges *et al.* 1985; Nebeker *et al.* 1993; Byers 1995). At high densities, the beetles can overcome tree defences through mass attacks mediated through aggregation pheromones. In the colonisation process, SPB introduces several fungi into its host tree. At least one fungal associate, *Ophiostoma minus* (Hedge.) H. and P. Sydow, might play an important role in exhausting host defences (Klepzig *et al.* 2005), destroying phloem and cambium, and enabling beetle development therein (Six and Klepzig 2004).

In addition to *O. minus*, SPB females also utilise highly evolved, specialised structures, called mycangia (singular mycangium), to house and nurture symbiotic fungi *Entomocorticium* sp. *A* Hsiau and Harrington and *Ceratocystiopsis ranaculosus* J.R. Bridges & T.J. Perry (Happ *et al.* 1971; Barras and Perry 1972; Barras and Taylor 1973; Hsiau and Harrington 2003). These fungi (like many mycangial fungi) are only weakly virulent in their host trees (Ross *et al.* 1992; Harrington 1993a,b). These mycangial fungi provide nutrients for developing larvae under bark (Barras 1973; Bridges 1983; Goldhammer *et al.* 1990; Coppedge *et al.* 1995; Six and Klepzig 2004). Within Scolytine beetles, mycangia are most often located on the exterior surface (rarely in the oral cavity) of female pronotum (Batra 1963; Whitney and Farris 1970; Barras and Perry 1971, 1972; Happ *et al.* 1971; Happ *et al.* 1976; Wood 1982; Furniss *et al.* 1987; Lanier *et al.* 1988; Levieux *et al.* 1991; Kajimura and Hijii 1992; Paine *et al.* 1997; Six 2003; Stone *et al.* 2007). Some mycangia are associated with glandular cells, and secretions from these specialised

cells are thought to serve to protect mycangial fungal cells from desiccation, provide nourishment for proliferating fungal propagules, regulate fungal species composition and determine the form of fungal growth in mycangia (Batra 1963; Abrahamson *et al.* 1967; Francke-Grossmann 1967; Schneider and Rudinsky 1969a,b; Barras and Perry 1971; Happ *et al.* 1971; Six 2003).

Despite the importance of these mycangial fungi in beetle biology, detailed ultrastructural observations to understand the function of individual components of the mycangial system are still lacking for SPB and many other tree-killing bark beetle species. Particularly, the mechanism by which fungal growth and morphology are regulated in the mycangium is not known. We have been exploring the ultrastructure of the mycangium and its function in SPB (Pechanova *et al.* 2008; Scott *et al.* 2008) and provide herein a more detailed visualisation of the ultrastructure of the mycangial system in the female SPB. On the basis of the current morphological studies and previously reported studies, we present some potential mechanisms by which SPB might maintain this complex symbiotic system.

Materials and Methods

SPB-infested loblolly pine (*Pinus taeda* L.) sites were located in the National Forests in Mississippi – Spring 2005: DeSoto Ranger District near Hattiesburg (Mississippi, USA); Summer 2008: Homochitto Ranger District near Meadville (Mississippi, USA). Bolts (80 cm long) from the lower bole of 20 trees were cut and carried to the laboratory. Some bolts were placed in rearing cages, and others were dissected immediately. Dissection yielded large numbers of larvae in different developmental stages from 1st instar to 4th instar. Larvae were placed in individual vials. Emerging adult beetles were collected daily and placed in individual vials. All vials were kept in a refrigerator (4°C) until analysis. Adult beetles were sexed under a microscope based on the presence (female) or absence (male) of a pronotal mycangium (Wood 1982) and the presence (in males) of a groove on the head. Larvae were not sexed.

Beetles (75 adults and 75 larvae) and galleries (30) were fixed in half-strength Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde) with phosphate buffer (0.1 M, pH 7.2) for 48 h at 4°C (Karnovsky 1965). Beetles were then cryo-fractured in liquid nitrogen. Specimens were dehydrated through a graded ethanol series and stored in 100% ethanol. Specimens were subsequently critical point dried in a Polaron E3000 Critical Point Dryer (Quorum Technologies, Newhaven, UK) using liquid CO_2. The tissues were then mounted on stubs and coated with gold–palladium using a Polaron E5100 sputter coater (Quorum Technologies). Samples were examined with a JSM-6500F scanning electron microscope (JEOL, Tokyo, Japan). One light microscope image (Fig. 4E) included in this manuscript is taken from previous (unpublished) work by T. Perry and S.J. Barras, USDA Forest Service, Pineville, LA (details in Happ *et al.* 1971).

Results

Mycangial fungi in immature stages of SPB

Dissection of bolts yielded various immature stages of SPB. Beetles constructed galleries within the phloem (Fig. 1A) within which symbiotic fungi were evident. Larvae were frequently found near and on masses of fungal hyphae (Fig. 1B–D). Yeast-like growth was also observed within larval galleries (Fig. 1C,D). Signs of possible fungal feeding by larvae were also present (eg fungal cells in the foregut of five larvae; Fig. 1E,F). Based on morphology, it appeared that the larvae may have fed on at least two species of fungi (Fig. 1G) which could be *Entomocorticium* sp. *A* and *C. ranaculosus* based on their spore formation. However, because these two fungi do not readily sporulate on artificial media, it is difficult to identify them based on spore morphology. Morphologically similar fungal cells were also detected in the midgut of 18 larvae along with digested material (Fig. 1H).

Mycangial system in the female SPB

Parallel to the earlier reports (Happ *et al.* 1971; Barras and Perry 1972), we located a pair of mycangia at the anterior region of the pronotum of female SPB (Fig. 2A). The two mycangia, one on each side of the prothorax, are terminated on the dorsal side at a 'mycangial bridge' (see Fig. 5 for visualisation). However, this sclerotised structure did not appear to provide a passage between the two mycangia (Fig. 2B), suggesting that each mycangium functions independently.

Profuse fungal growth, mainly in the form of columns of spore-like cells, was frequently observed in the mycangial lumen (Fig. 2C–E,). These fungal cell masses were covered in what appeared to be a thin layer of membranous material, seemingly holding the fungal cells together. We also observed amorphous, fluidic material filling the gaps between fungal cells and, perhaps, further promoting the integrity and/or adhesion of the cell mass. Such substances appeared to be secreted (or at least emanating) from surrounding cells (perhaps the gland cells reported in Happ *et al.* 1971) into the mycangial lumen via abundant ductules (Fig. 2F,G).

We observed that the mycangial lumen and gland cells were surrounded by abundant tracheoles (Fig. 2H,I). These tracheoles were connected to the mycangial lumen, through the beetle's exoskeleton, via small openings (<1 μm) on the exterior of the beetle's prothorax (Fig. 2J–M).

Previously undescribed structures present surrounding the mycangium

Sagittal views revealed that the elongate mycangium was surrounded by an inner wall and anterior fold (Fig. 3A).

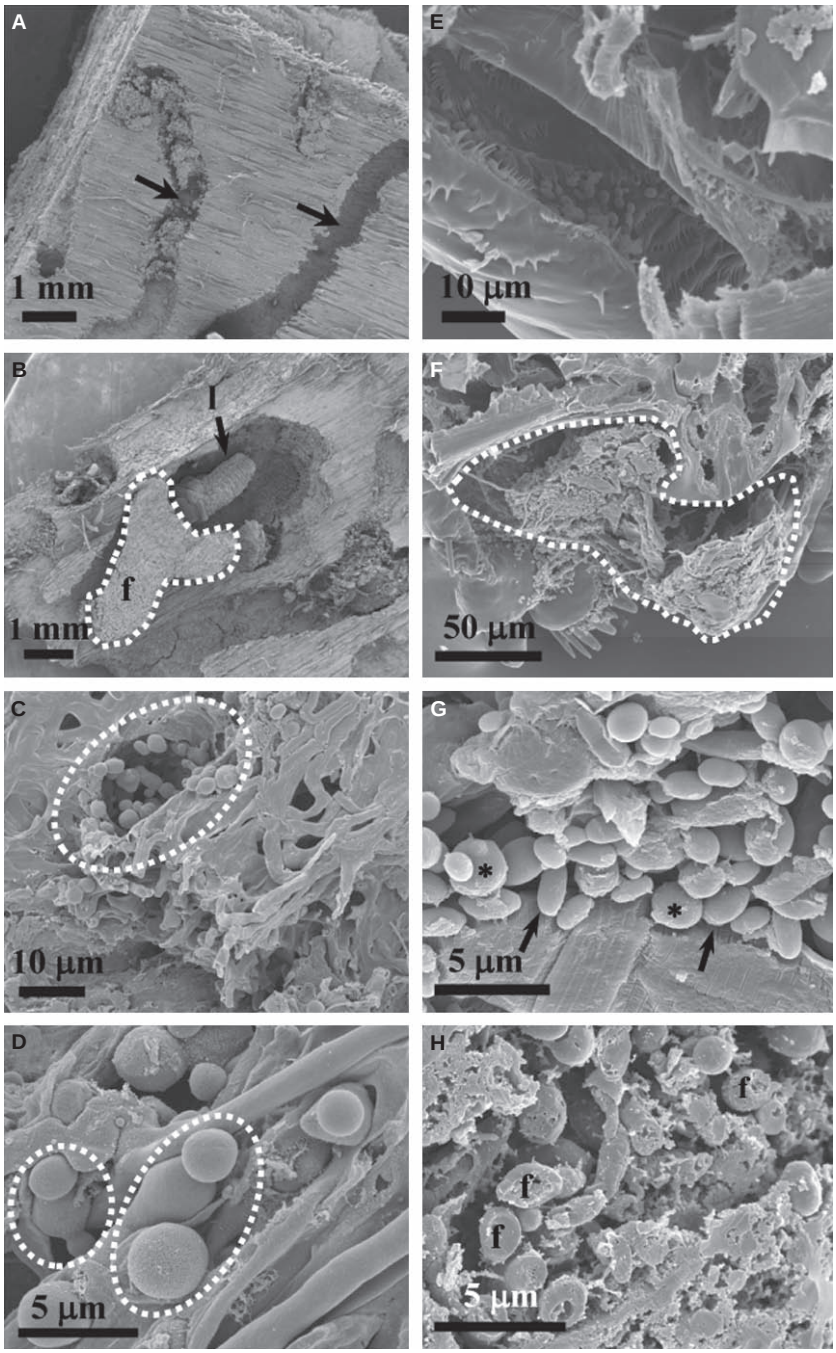

Fig. 1— The southern pine beetle larvae, larval galleries and mutualistic fungi. —A. Two different larval galleries (arrows) within the phloem. —B. Larva (l) feeding on fungus-infested phloem tissues (f; dotted area). —C, D. Close-up views of the dotted area in B showing yeast-like fungal growth. —E, F. Two different views showing fungal cells in the foregut of a southern pine beetle larva. —G. Close-up view of the dotted area in F showing two different fungal cells. Asterisk and arrow show two different kinds of fungal cells. —H. Fungal cells in the midgut of larva (f).

Previously unknown pore-like depressions (or pits) lined up along with the mycangium were present in both the inner wall and anterior fold (Fig. 3B–E). Each pit had an average length and width of 0.8 ± 0.48 μm (mean ± standard deviation) and 0.7 ± 0.37 μm, respectively (n = 10). These pits overlaid the mycangial gland cells and tracheoles within the gland cells. An unknown secretory product was often observed in and around the pits (Fig. 3D,F). Similar structures were also observed, less frequently, on the walls of the mycangial lumen (Fig. 3G,H).

Fungal entrance into and release from the mycangial lumen to beetle galleries

While we observed evidence for propagule germination and hyphal growth near pits in the inner wall and anterior fold

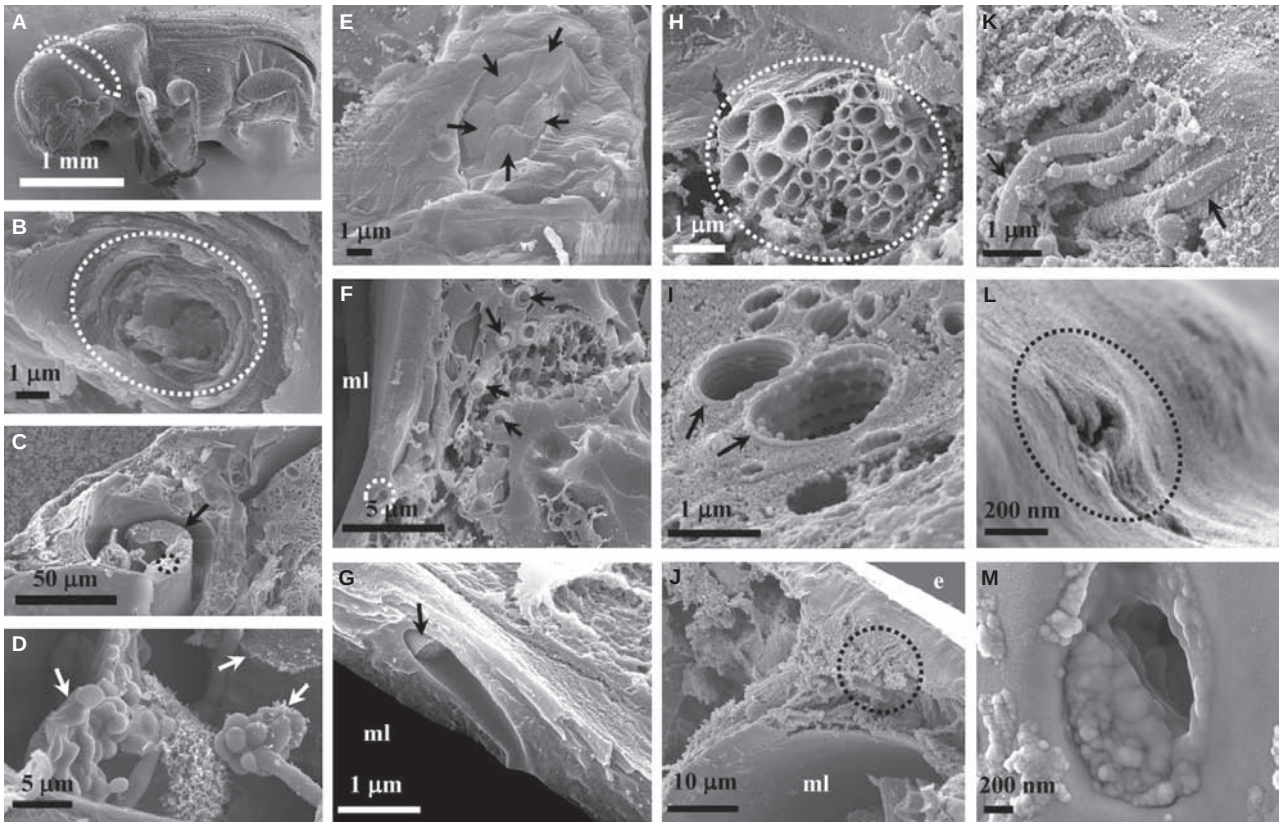

Fig. 2— The mycangial system of the southern pine beetle. —A. One of the pairs of mycangia at the anterior region of the pronotum (dotted line) of a female beetle. —B. A 'mycangial bridge' (circled with dotted lines) on the dorsal side of beetles joining two mycangia on each side of the prothorax. —C, D. Spore-like fungal cells (arrows) in the mycangial lumen. —E. Close-up view of the dotted area in C showing fungi growing in yeast-like form (arrows). —F. Gland cells and ductules (arrows) surrounding the mycangial lumen (ml). —G. Close-up view of the dotted area (gland cells and ductules) in F showing secretory product (arrow) channelled to the mycangium lumen (ml). —H. Tracheoles (circled area) surrounding the mycangial lumen within the gland cells. —I. Close-up view of tracheoles (arrows). —J. Dorsal view of a bundle of tracheoles (dotted line) close to the mycangial lumen (ml) is connected to the exterior (e) of the beetle prothorax. —K. Close-up view of tracheoles (arrows) in dotted area in J. —L. Connection of the tracheoles to an opening (dotted line) on the exterior of the beetle's prothorax (sagittal view). —M. Close-up view of the exterior opening.

(Fig. 4A,B), we did not notice any hyphal growth from the inner wall or anterior fold to the mycangial lumen nor within the ductules surrounding the mycangium. The structures observed were <500 nm in diameter (Fig. 4C), too small to be fungal hyphae. We often observed abundant fungal cells in the opening of the mycangium between the inner wall and anterior fold of the mycangium (Fig. 4D).

We did not find any direct evidence of fungal release from the mycangium into the ovipositional galleries. However, images showed what appeared to be fungal spores bundled together in a membranous structure within the mycangial lumen (Fig. 4E) and similar bundles within sections of larval galleries (Fig. 4F–N).

Discussion and Conclusion

Our detailed ultrastructural observations of mycangia and galleries of SPB provide further evidence for the mutualistic interactions between SPB and its mycangial fungi. Although the current study did not find how fungi are released from the mycangia, it is highly likely that adult female beetles introduce their mutualistic fungi during the process of host selection and concentration of conspecific attacks as fungal growth is observed within days after bark beetle host colonisation (Paine *et al.* 1997). Our results further demonstrate that fungal cells are bundled together in a membranous structure within the mycangial lumen and possibly introduced as a bundle during gallery excavation because we often observed similar structures within the sections of egg niches and larval galleries. We have no knowledge of how these membranous structures form. It is possible that they may form as a result of secretions from the mycangial gland cells.

We often observed fungal cells both in the foregut and in the midgut of larvae, indicating that larvae are feeding on fungi either through direct consumption or through feeding on fungal-infested host tissue. Further, morphological

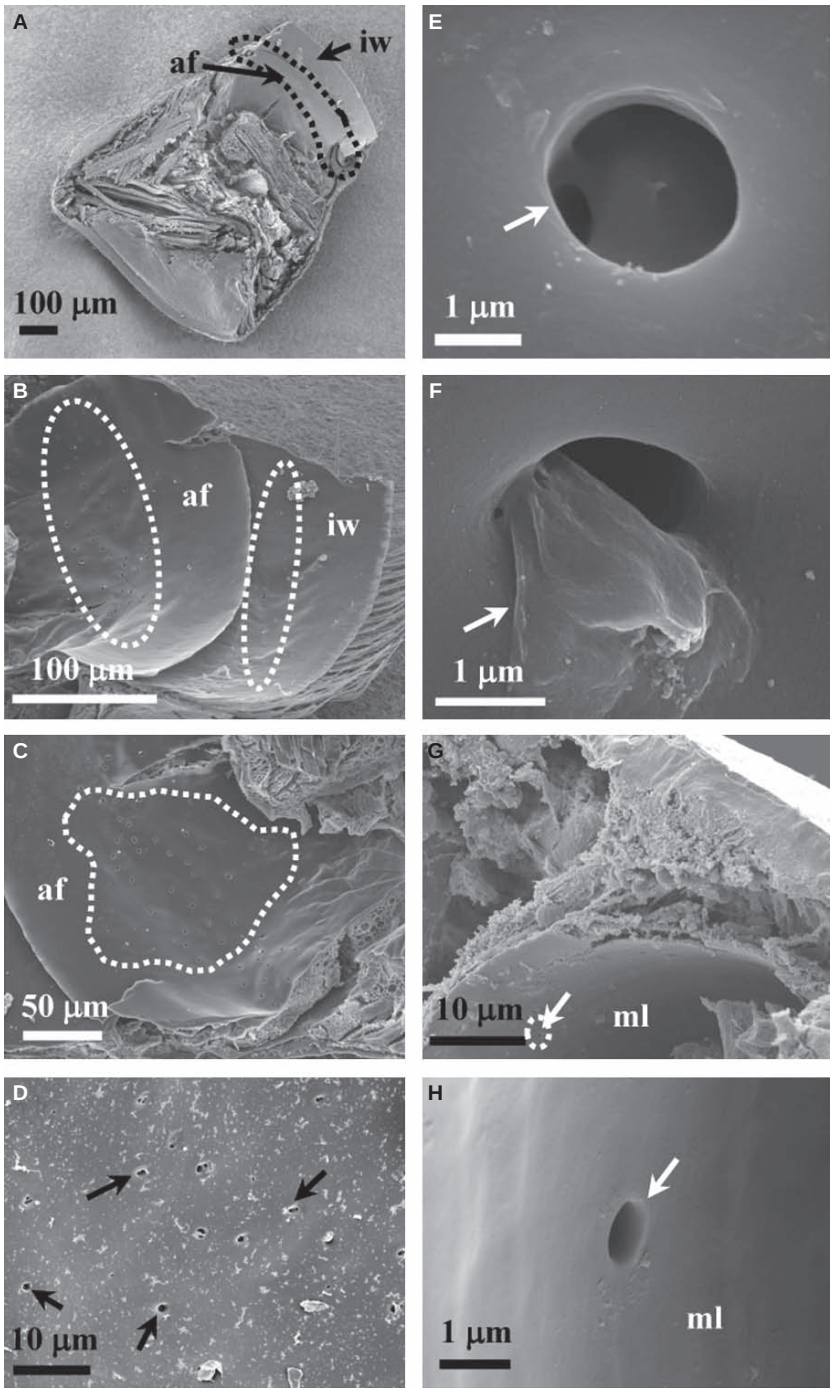

Fig. 3— A new structure found surrounding the mycangium. —A. The elongate mycangium (dotted line) is surrounded by the inner wall (iw) and the anterior fold (af) in a sagittal view of the left half of the pronotum. —B, C. Both the inner wall (iw) and the anterior fold (af) have pore-like structures (dotted area). —D, E. Close-up view of pore-like structures (arrows) in the anterior fold. —F. Secretory products filling one of the pore-like structures (arrow) in the inner wall. —G. A few pore-like structures (dotted line with an arrow) are also present on the walls of the mycangial lumen (ml). —H. Close-up view of pore-like structures (arrow) in G.

differences in these fungal cells in larval guts suggest that larvae may feed on at least two species of fungi, possibly *Entomocorticium* sp. *A* and *C. ranaculosus*. However, because they do not readily sporulate on artificial media, it is difficult to identify them based on spore morphology. We speculate that beetle larvae may benefit from fungal feeding at least in two ways. In addition to obtaining nutrients from digested wood tissues, the fungi may also provide essential nutrients, such as nitrogen and sterols (Paine *et al.* 1997; Bentz and Six 2006; Bleiker and Six 2007; Klepzig *et al.* 2009). There is evidence for digested material in the larval midgut. The fungi or other associates may also alter the nutritional quality of the host tissue (Hodges *et al.* 1968; Barras and Hodges 1969; Bridges 1981). As it has been widely suggested that the same fungi

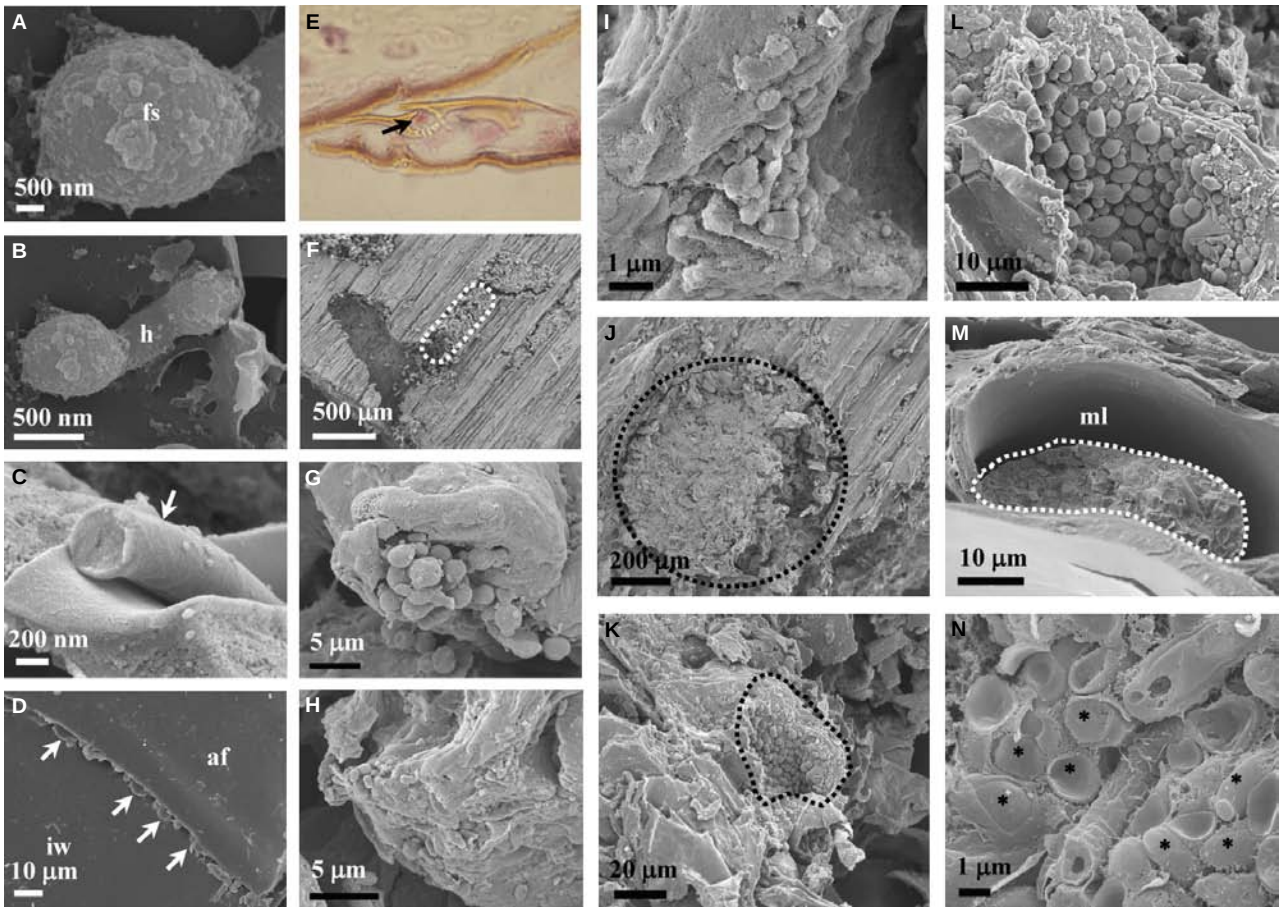

Fig. 4— Fungal entrance into and release from the mycangial lumen into beetle galleries. —A. A fungal propagule (fs) germinates in the inner wall of prothorax. —B. Hypha (h) growing near a pore-like structure. —C. Solid structures (arrow) in a ductule within the gland cells surrounding mycangia. —D. Fungal cells (arrows) in the opening of a mycangium between the inner wall (iw) and anterior fold (af). —E. Bundled fungal cells (arrow) in the mycangium. —F. Accumulation of structures (dotted line) in larval galleries. —G—I. Close-up view of dotted area in F with fungal cells. —J. A single egg niche (circled) within the phloem. —K. Similar formations as in F and G in egg niches in J. —L. Close-up view of the dotted area in K shows fungal cells inside these structures. —M. A mycangial lumen (ml) in which fungal cells are bundled together (dotted area). —N. Close-up view of dotted area in M showing fungal cells (asterisks).

may also help beetle larvae to digest and metabolise plant secondary compounds from their host tissues (Dowd 1992; Despres *et al.* 2007). Whether through direct consumption of fungi or feeding on fungal-modified host tissue, SPB developing in the presence of mycangial fungi are larger and more fecund than those that develop in the absence of the fungi (Barras 1973; Goldhammer *et al.* 1990; Coppedge *et al.* 1995).

Our study clearly indicated that when beetles emerge from parental hosts, they often carry mutualistic fungi within their mycangia, consistent with the previous reports (Barras 1973; Hofstetter *et al.* 2006). Introduction of these fungi into the mycangia most likely occurs after adults emerge from pupal chambers (Whitney *et al.* 1987).

The mechanism by which mycangial fungi are maintained in the yeast-like form we observed in the mycangia is not known. It has been suggested that chemical secretions from gland cells surrounding mycangia (in general) may regulate fungal growth and morphology (Batra 1963; Abrahamson *et al.* 1967; Francke-Grossmann 1967; Schneider and Rudinsky 1969a,b; Barras and Perry 1971; Happ *et al.* 1971). We observed the possible secretion of substances from gland cells into the SPB mycangial lumen. Based on this and previously published data, we speculate that these substances may be responsible for maintaining the observed fungal growth (yeast-like growth) and morphology. Scott *et al.* (2008) recently suggested that secretion of a novel antibiotic (mycangimycin) from actinomycete bacteria found within the SPB mycangium is likely involved in selection and growth of mycangial fungi. Such antibiotic selectively inhibited the growth of antagonistic fungi (eg *O. minus*), but not the symbiotic mycangial fungi (eg *Entomocorticium* sp. *A*).

We observed previously unknown pore-like depressions (or pits) lined up along with the mycangium in both the inner wall and anterior fold surrounding the mycangium (Fig. 5). These newly discovered pits contained some substances that are likely secreted by the underlying gland cells to the surface of the inner wall and anterior fold. Although neither function nor content of these chemical substances have been reported, they may regulate the species composition of fungi in the mycangium. However, these chemical substances may contain mycangimycin secreted from the symbiotic actinomycete bacterium within the SPB mycangium (Scott *et al.* 2008). Selection of fungi entering the mycangium might occur on these surfaces in the presence of secreted chemicals. Spores of the symbiotic fungi *Entomocorticium* sp. *A* and/or *C. ranaculosus* might be the only ones that can germinate and grow into the mycangial lumen. Frequent observations of either fungus in the mycangial lumen support this suggestion. We do not dismiss the possibility that the secreted chemicals might deter parasites (eg nematodes) or pathogenic microorganisms (eg fungi and bacteria) from entering the mycangium.

Further, the secreted chemicals might also be involved in germination of symbiotic fungal spores near the mycangial opening, followed by a hyphal growth into the mycangial lumen. However, we did not observe hyphal growth into the mycangial lumen, suggesting that either these chemicals do not play any role in hyphal growth or special conditions, such as certain pH level, temperature or precipitation, may be required for spore germination and growth.

Abundant tracheoles were observed within the mycangial system of SPB, which may allow large amount of gas exchange by transporting oxygen into the mycangial lumen and surrounding gland cells and by transporting carbon dioxide out of prothorax. This system of gas exchange may also affect growth and sporulation (Marmiroli *et al.* 1983; Miller 1989; Treinin and Simchen 1993; Griffin 1994; Codon *et al.* 1995). The tracheoles within the SPB mycangial system are connected to small openings on the surface of prothorax. Abundant air movement into the mycangial system could promote fungal sporulation and support high metabolic activities in gland cells for the production of proteins and chemicals (Stone *et al.* 2007; Pechanova *et al.* 2008).

In conclusion, the female SPB have developed a mycangial system (including the newly discovered pits) that appears to be involved in maintenance, and perhaps selective growth, of symbiotic fungi in yeast-like growth and potentially provides aeration via tracheoles for fungal reproduction. Mycangial structure (eg inner wall, anterior fold, mycangial lumen, tracheoles) also may play a role in intake and release of fungal spores into beetle galleries where fungi provide essential nutrients for larvae.

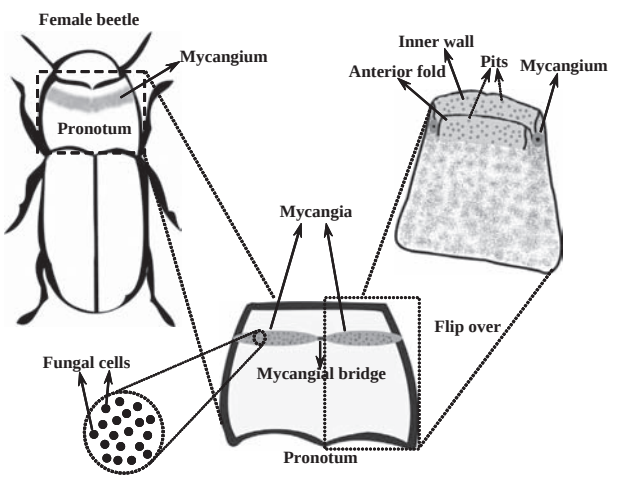

Fig. 5— Schematic visualisation of the female southern pine beetle showing locations of the mycangium, mycangial bridge, fungal cells and the newly discovered structures surrounding the mycangium in a sagittal view of the pronotum.

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