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Histopathology of Laboulbeniales (Ascomycota: Laboulbeniales): ectoparasitic fungi on ants (Hymenoptera: Formicidae)

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Abstract

Among the many associations between fungi and ants, the associations involving the ectoparasitic fungi Laboulbeniales (Ascomycota: Laboulbeniales) have remained largely enigmatic even today. However, for two of the six ant-parasitizing Laboulbeniales, it has been found that parasitism is correlated with diminished survival of their hosts, especially under resource limitation. In the present study, we investigate whether these fitness impacts are linked to an intrusion into the body cavity by the ectoparasites. Light, scanning and transmission electron microscopy were used to study the mode of attachment and the presence of penetrating structures in four of the six currently recognized ant-parasitizing Laboulbeniales. No indication of penetration was found, suggesting that the reported fitness impacts are not linked to an intrusion into the body cavity. A better understanding of host-parasite interactions involving Laboulbeniales on ant hosts is necessary, considering that Laboulbeniales parasitizing ants impact their hosts' fitness and that monitoring studies have revealed that an infection with Laboulbeniales is much more common in European ants than previously thought.

Key words: Host-parasite interaction, symbiosis, haustoria, nutrition, invasive species, ant-fungus association.

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Introduction

Ants (Hymenoptera: Formicidae) are well-known to entertain a variety of associations with diverse organisms (Hölldobler & Wilson 1990). Among these organisms, fungi cover the whole spectrum of relationships from mutualism to parasitism (Vega & Blackwell 2005). While some ant-fungus associations are comparably well studied, e.g., in the tribe Attini (Hymenoptera: Formicidae: Myrmicinae: Attini) (Mehdiabadi & Schultz 2010), other associations, e.g., involving the fungi of the order Laboulbeniales (Ascomycota: Laboulbeniales) have received less attention. Laboulbeniales are obligate biotrophic fungi living as ectoparasites mostly on insects (Weir & Blackwell 2005) with currently six different species from three genera described on ant hosts (Santamaria & Espadaler 2014).

A common view is that Laboulbeniales have little or no effect on parasitized hosts (Benjamin 1971, Tavares 1985, Kaur & Mukerji 2006). This is likely in part due to the existence of only few controlled studies investigating fitness effects of Laboulbeniales (Whisler 1968, Strandberg & Tucker 1974). Early notes on Laboulbeniales parasitizing ants also report contradictory results. For example, Wheeler (1910) noted the behaviour of *Lasius neoniger* Emery, 1893 ants to be unaffected by *Laboulbenia formicarum* Thaxter, 1902 but also that parasitized colonies seemed less prosperous than non-parasitized colonies (for similar observations, see also Rick 1903, Smith 1917). Recently, possible fitness effects of Laboulbeniales parasitizing ant hosts have been addressed more explicitly. Two species, *L. formicarum* and *Rickia wasmannii* Cavara, 1899 parasitizing Formicinae and Myrmicinae ant species, respectively, have been found to reduce the survival of their European ant hosts (Csata & al. 2014, Konrad & al. 2015). Furthermore, although the potentially interacting factors of individual ant age and infection level were not disentangled, mortality was found to be more pronounced under resource limitation, i.e., food and / or water restriction (Báthori & al. 2015a, Konrad & al. 2015).

Generally, Laboulbeniales are believed to draw resources from their hosts for growth and development, either through absorption or via contact with living tissues (Scheloske 1969, Benjamin 1971, Tavares 1985). Although some Laboulbeniales appear to only attach to the integument of their hosts, others produce penetration structures (reviewed in Scheloske 1969, Benjamin 1971, Tavares 1985, see also Tab. 1). Invasion into the body cavity in the

Tab. 1: Overview of current literature indicating the presence of penetration structures in species of ectoparasitic Laboulbeniales (Ascomycota) together with arthropod host species that were examined, the type of evidence given (photographs, histological sections, drawings, reports or indirect evidence through staining) and reported fitness impact (yes, no, unknown, together with type of evidence given and related literature reference). Superscript small letters indicate: [a] described as *Paracoreomyces thaxteri*, [b] described as *Herpomyces tricuspidatus* see BENJAMIN (1971: p. 33), [c] according to TAVARES (1979) the structure could also simply be cracks in the cuticle, [d] described as *Sunius prolixus*, [e] Orifice is the passage for the penetration structure on the base of the foot of the thallus, [f] described as *Menopon pallidum*, [g] described as *Goniocotes abdominalis*, [h] *Herpomyces* spp. on *Parcoblatta lata*, [i] *Trenomyces* spp. on unspecified hosts, [j] Scheloske (1969) reported this species in the genus *Harpalus*.

Laboulbeniales species	**Host species (Order)**	**Reference**	**Evidence**	**Fitness impact**
Arthrorhynchus nycteribiae	*Penicillidia conspicua* (Diptera)	BLACKWELL (1980)	Fig. 2: photograph of penetration structure	unknown
Arthrorhynchus cyclopodiae	*Cyclopodia macrura* (Diptera)	THAXTER (1896)	Plate XLVIII Fig. 2: drawing of penetration structure	unknown
Coreomyces thaxteri[a]	*Stenocorixa protrusa* (Hemiptera)	POISSON (1929)	reports penetration structure	unknown
Coreomyces corisae	*Corixa affinis* (Hemiptera)	POISSON (1929)	reports penetration structure	unknown
Dimeromyces rhizophorus	unknown species (Diptera)	THAXTER (1908)	Plate XXVIII Figs. 6, 7: drawing of penetration structure	yes: report of injury to abdominal tissues (THAXTER 1908)
Gloeandromyces nycteribiidarum	*Pterellipsi aranea* (Diptera)	THAXTER (1931)	Plate XX Fig. 16: drawing of penetration structure	unknown
Herpomyces ectobiae	*Blattella germanica* (Blattodea)	TAVARES (1985)	Plate 10 b, c: histological sections of penetration structure	yes: higher mortality (GEMENO & al. 2004)[h]
Herpomyces ectobiae	*Blattella germanica* (Blattodea)	RICHARDS & SMITH (1956)	reported as similar to *H. stylopygae* on *B. orientalis*	yes and no: report of unaffected motility, reproduction and longevity, but damage of the epidermis (RICHARDS & SMITH 1956)
Herpomyces stylopygae	*Blatta orientalis* (Blattodea)	RICHARDS & SMITH (1956)	Figs. 16 - 23: histological sections of penetration structure (3 types: Ø 0.1 - 0.3, Ø 1.5 - 2 and Ø 2 - 5 µm)	see above (RICHARDS & SMITH 1956)
Herpomyces paranensis[b]	*Blaberus cranifer* (Blattodea)	RICHARDS & SMITH (1956)	reported as similar to *H. stylopygae* on *B. orientalis*	see above (RICHARDS & SMITH 1956)
Herpomyces paranensis	*Blaberus cranifer* (Blattodea)	TAVARES (1979)	Fig. 9.3: histological sections of penetration structure	see above (RICHARDS & SMITH 1956)
Hesperomyces virescens	*Chilocorus bipustulatus* (Coleoptera)	KAMBUROV & al. (1967)	Plate 2, Plate 4b: photographs of penetration structures (Ø 2.5 - 3 µm)	yes: reduced survival (KAMBUROV & al. 1967, RIDDICK 2010) no: unaffected metabolism (APPLEBAUM & al. 1971)
Hesperomyces virescens	*Cycloneda sanguinea* (Coleoptera)	TAVARES (1979)	Fig. 9.4: histological sections of penetration structures	unknown
Hesperomyces virescens	*Cycloneda sanguinea* (Coleoptera)	TAVARES (1985)	Plate 16 a - c: histological sections of penetration structure	unknown
Hesperomyces virescens	*Adalia bipunctata* (Coleoptera)	WEIR & BEAKES (1996)	Figs. 17, 18: photograph of penetration pore on cuticle (Ø 3 µm with Ø 1 µm penetration pore)	unknown
Hesperomyces virescens	*Olla v-nigrum* (Coleoptera)	WEIR & BEAKES (1996)		unknown
Hydrophilomyces reflexus	*Phaenonotum extriatum* (Coleoptera)	TAVARES (1985)	Plate 31e: photograph of penetration structure	unknown
Laboulbenia gyrinidarum	*Aulonogyrus concinnus* (Coleoptera)	THONGHINI (1913)	Fig. 10: drawing of penetration structure[c]	unknown

Laboulbenia blanchardii	*Demetrias monostigma* (Coleoptera)	CÉPÉDE (1914)	Plate VI, Figs. 4, 5, 7, 8, 10, 11: drawing of penetration structure (Ø 0.5 - 6 µm)	unknown
Laboulbenia fasciculata	*Leistus fulvibarbis* (Coleoptera)	POISSON (1929)	reports penetration structure	unknown
Laboulbenia vulgaris	unknown species (Coleoptera)	COLLA (1926)	Fig. 2c: drawing of penetration structure	unknown
Laboulbenia macrotheca	*Harpalus affinis* (Coleoptera)	SCHELOSKE (1969)	reports penetration structure	unknown
Laboulbenia coneglanensis	*Pseudoophonus rufipes*[j] (Coleoptera)	SCHELOSKE (1969)	infers penetration structure from successful staining	unknown
Laboulbenia ophoni	*Ophonus puncticollis*[j] (Coleoptera)	SCHELOSKE (1969)	infers penetration structure from successful staining	unknown
Laboulbenia ophoni	*Ophonus rupicula*[j] (Coleoptera)	SCHELOSKE (1969)	infers penetration structure from successful staining	unknown
Laboulbenia rougetii	*Brachinus crepitans* (Coleoptera)	SCHELOSKE (1969)	infers penetration structure from successful staining	unknown
Microsomyces psammoechi	*Psammoechus orbicollis* (Coleoptera)	THAXTER (1931)	Plate XXXIX Figs. 1, 2: drawing of penetration structure	unknown
Moschomyces insignis	*Astenus prolixus*[d] (Coleoptera)	THAXTER (1896)	Plate XI Fig. 16: drawing of penetration structure	unknown
Rhizomyces ctenophorus	*Diopsis thoracica* (Diptera)	THAXTER (1908)	Plate IV Fig. 3: drawing of penetration structure	unknown
Rhizomyces gibbosus	*Diopsis* sp. (Diptera)	THAXTER (1908)	Plate LII Fig. 23: drawing of penetration structure	unknown
Stigmatomyces scaptomyzae	*Scaptomyza graminum* (Diptera)	DAINAT & MAINER (1974)	Figs. 1 - 6: histological sections of penetration structure (Ø 2.5 - 4 µm with Ø 1 µm penetration pore) and of orifice[e]	unknown
Stigmatomyces baerii	*Fannia canicularis* (Diptera)	BOEDIJN (1923)	Figs. 6, 7: drawing of penetration structure and of orifice[e]	unknown
Stigmatomyces ceratophorus	*Fannia canicularis* (Diptera)	WHISLER (1968)	Figs. 2 - 4: photograph of orifice[e] and penetration pore on cuticle	no: survival experiments (WHISLER 1968)
Stigmatomyces ceratophorus	*Fannia canicularis* (Diptera)	TAVARES (1985)	Plate 16 d: histological sections of penetration structure	unknown
Trenomyces histophtorus	*Menopon* sp. (Phthiraptera)	TAVARES (1979)	Fig. 9.7: photograph of penetration structure	yes: report of fat body reduction (EICHLER 1951)[i]
Trenomyces histophtorus	*Menopon gallinae*[f] (Phthiraptera)	CHATTON & PICARD (1909)	report of penetration structure	yes: report of fat body reduction (CHATTON & PICARD 1909)
Trenomyces histophtorus	*Goniodes gigas*[g] (Phthiraptera)	CHATTON & PICARD (1909)	report of penetration structure	yes: report of fat body reduction (CHATTON & PICARD 1909)
Trenomyces histophtorus	*Menopon gallinae* (Phthiraptera)	MEOLA & DEVANEY (1976)	Figs. 5 - 7: histological sections of penetration structure (Ø 3.5 µm)	yes: loss of fat body and skeletal muscle (MEOLA & DEVANEY 1976)
Trenomyces histophtorus	*Lipeurus caponis* (Phthiraptera)	MEOLA & DEVANEY (1976)	report of penetration structure	see above
Trenomyces histophtorus	*Menacanthus stramineus* (Phthiraptera)	MEOLA & DEVANEY (1976)	report of penetration structure	see above
Trenomyces histophtorus	*Menacanthus stramineus* (Phthiraptera)	MEOLA & TAVARES (1982)	Figs. 1 - 9: histological sections of penetration structure	yes: report of degeneration of host cells (MEOLA & TAVARES 1982)

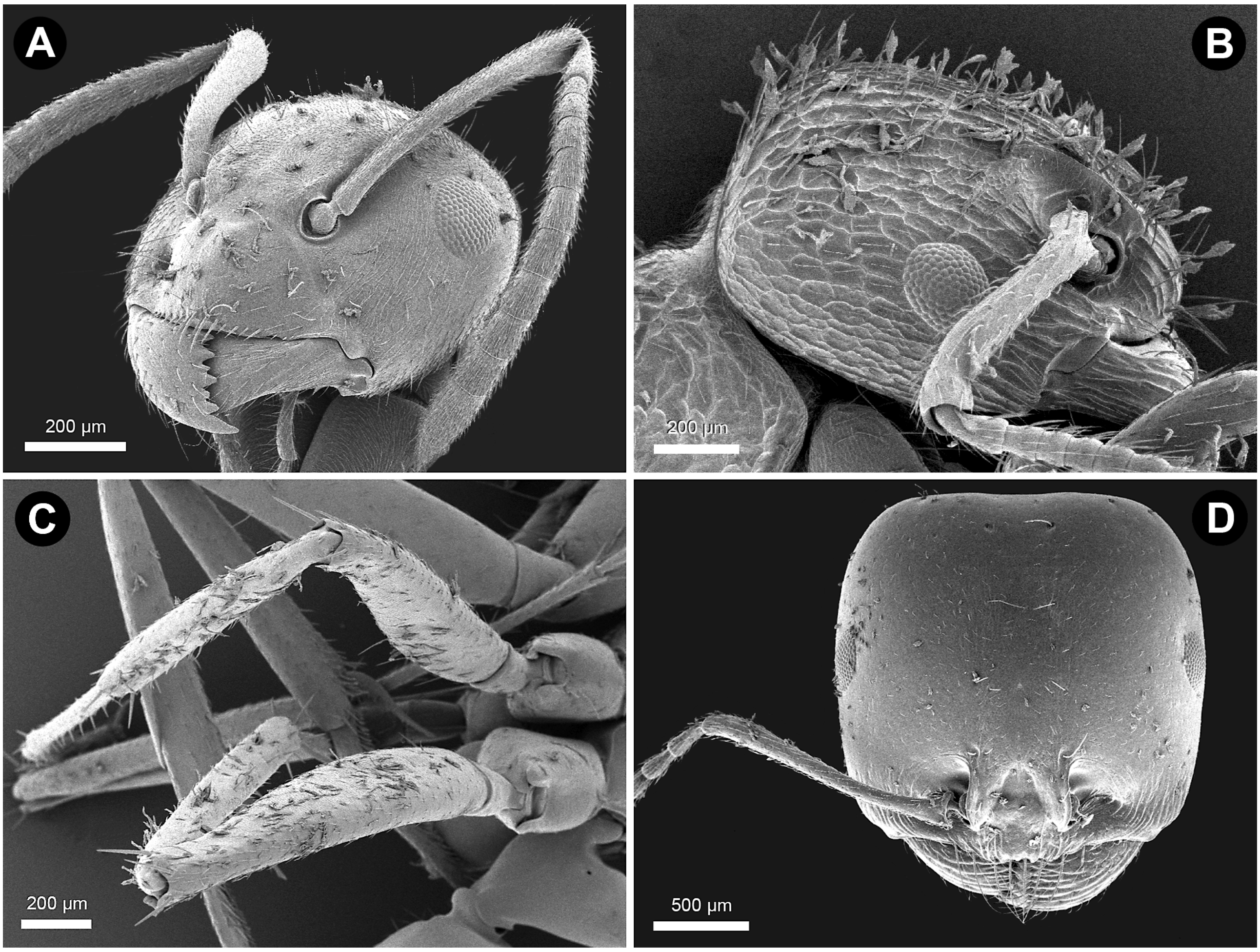

Fig. 1: Overview of Laboulbeniales parasitizing the ants *Lasius neglectus* (A), *Myrmica scabrinodis* (B), *Camponotus sylvaticus* (C, showing mid- and hindlegs) and *Messor wasmanni* (D) seen under scanning electron microscopy. The fungal thalli of the Laboulbeniales appear as bristle-like structures attached to the cuticle.

latter species can be more or less pronounced. Some invade the cuticle only superficially (THONGHINI 1913, COLLA 1926), while others form a bulb in the epidermal cell layer without further intrusion into the body cavity (RICHARDS & SMITH 1956). Distinct Laboulbeniales even completely intrude into the body cavity, e.g., *Hesperomyces virescens* THAXTER, 1891 parasitizing coccinellids (KAMBUROV & al. 1967, TAVARES 1979, WEIR & BEAKES 1996) or *Trenomyces histophtorus* CHATTON & PICARD, 1909 parasitizing lice (MEOLA & DEVANEY 1976, BLACKWELL 1980).

Although tentative, so far, a clear link between the intrusion of Laboulbeniales into the body cavity and the degree of impact on their hosts has not been established (Tab. 1). In light of recent evidence that Laboulbeniales negatively affect the survival of ant hosts, especially under resource limitation, in the present study we investigate the attachment and presence of cuticular penetration in ant-fungus associations involving Laboulbeniales. Histopathological studies were performed on four European Laboulbeniales that parasitize ants, namely *Laboulbenia formicarum* parasitizing the invasive garden ant *Lasius neglectus* VAN LOON, BOOMSMA & ANDRÀSFALVY, 1990, *Rickia wasmannii* parasitizing different *Myrmica* LATREILLE, 1804 species, *Laboulbenia camponoti* BATRA, 1963 on the ant *Camponotus sylvaticus* (OLIVIER, 1792) and *Rickia lenoirii* SANTAMARIA, 2015 on *Messor wasmanni* KRAUSSE, 1910.

Materials and methods

Workers of four European ants parasitized by Laboulbeniales were compared. *Lasius neglectus* parasitized by *Laboulbenia formicarum* collected in Gif-sur-Yvette, near Paris, France (48° 42' 19.70" N, 2° 07' 56.30" E) in 2012, *Myrmica scabrinodis* NYLANDER, 1846 parasitized by *Rickia wasmannii* collected near Meszes, Hungary (48° 27' 08.39" N, 20° 49' 25.18" E) in 2013, *Camponotus sylvaticus* parasitized by *Laboulbenia camponoti* collected in La Selva del Camp, Terragona, NE Spain (41° 13' 07" N, 1° 08' 35" E) in 2012 and *Messor wasmanni* parasitized by *Rickia lenoirii* collected in Greece, Aegina Island (37° 45' N, 23° 26' E) in 2009.

For light microscopy (LM) and transmission electron microscopy (TEM), anterior head, anterior and posterior thorax, and posterior abdomen parts were fixed during 12 h in cold 2% glutaraldehyde, buffered at pH 7.3 with 50 mM Na-cacodylate and 150 mM saccharose. One hour after postfixation in 2% osmium tetroxide in the same buffer, tissues were dehydrated in a graded acetone series, embedded in Araldite and sectioned with a Leica EM UC6 microtome. Serial semithin 1 µm sections were stained with methylene blue and thionin and viewed in an Olympus BX-51 microscope, double stained 70 nm ultrathin sections were

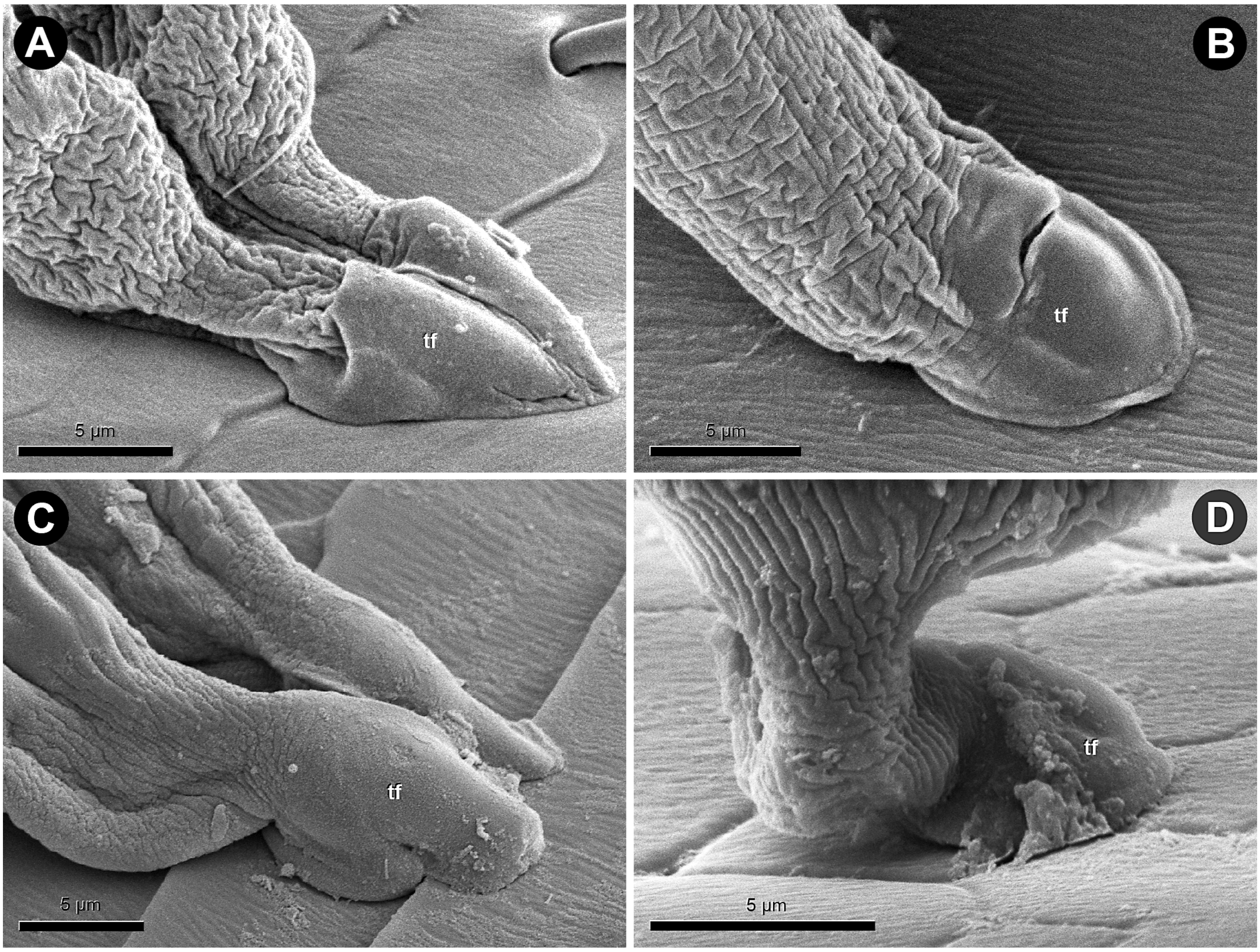

Fig. 2: Attachment of the Laboulbeniales on ants: (A) *Rickia wasmannii* on *Myrmica scabrinodis*, (B) *Laboulbenia formicarum* on *Lasius neglectus*, (C) *Laboulbenia camponoti* on *Camponotus sylvaticus*, and (D) *Rickia lenoirii* on *Messor wasmanni*. The fungi attach to the cuticle of their hosts via a hoof-like thallus foot structure (tf).

examined in a Zeiss EM900 electron microscope. Due to the inability to get fresh material necessary for LM and TEM sample preparation, LM and TEM was only performed for the ant hosts *Lasius neglectus* and *Myrmica scabrinodis*. For scanning electron microscopy (SEM), we examined both untreated infected ants and ants on which fungal thalli had been removed using a Minutien pin in order to see whether the ant integument was modified underneath the attachment site of the thalli. The material for SEM was mounted on stubs, gold coated and examined in a JEOL JSM-6360 scanning microscope.

Results

All Laboulbeniales on the ants *Lasius neglectus*, *Myrmica scabrinodis*, *Camponotus sylvaticus*, and *Messor wasmanni* (Fig. 1) attach to the host integument via a hoof-like foot structure (Fig. 2). Light microscopy and transmission electron microscopy observations of sectioned parasitized *L. neglectus* and *M. scabrinodis* ants never gave any trace of structures penetrating the integument (Figs. 3A - C; number of ant parts examined: *L. neglectus*: six heads, one posterior thorax and four abdomens; *M. scabrinodis*: three heads, one anterior thorax, one posterior thorax and one abdomen). The absence of penetration structures was confirmed by the absence of a fungal penetration pore underneath dislodged fungal thalli from all four host ants (Figs. 3D, E; Fig. S1 as digital supplementary material to this article, at the journal's web pages; confirmed under at least three dislodged thalli from eight *L. neglectus* workers, three *M. scabrinodis* workers, four *C. sylvaticus* workers and two *M. wasmanni* workers). Examination of host integument underneath removed fungal thalli using SEM revealed that the micro-sculpture of the integument appeared in all cases completely intact and unaffected, apart from two distinct remnants of the fungal foot structure. These remnants consisted of a horseshoe shaped outer ring that corresponded in size to the hoof-like foot structure (see Fig. 2A and Fig 3D for comparison; imprint measurements of *Rickia wasmannii* on *M. scabrinodis*; mean ± s.e. length along a line halving the shape symmetrically: 6.58 ± 0.32 µm; mean ± s.e. width along a line halving the shape asymmetrically: 5.8 ± 0.35 µm; N = 6) and a circular inner ring (mean ± s.e. length and width: 2.14 ± 0.19 and 1.88 ± 0.13 µm, respectively; N = 5). No indication was found in any case that the fungal foot structures were placed above particular features of the integument such as duct openings of gland cells or pores from wax channels.

Discussion

This study found no evidence that four of the currently six recognized ant-parasitizing fungi Laboulbeniales produce penetration structures into the body cavity of their hosts.

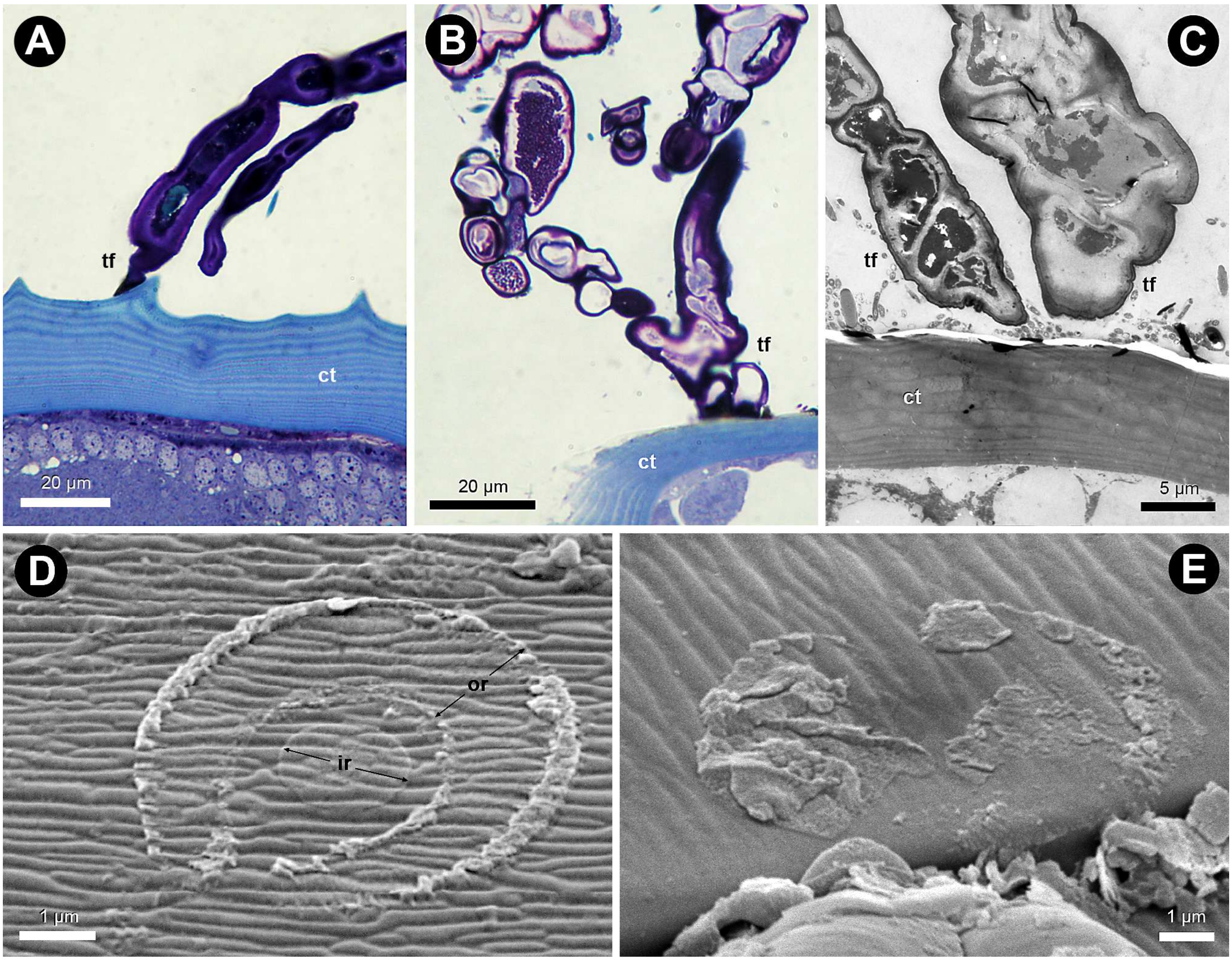

Fig. 3: Examination of sectioned parasitized ants under light (A, B) and transmission electron microscopy (C) did not reveal any indication of penetration structures originating from the thallus foot structure (tf) attached to the cuticle (ct). The absence of fungal penetration is confirmed through the experimental removal of fungal thalli from the cuticle (D, E). The cuticle under dislodged thalli only reveals peculiar imprints consisting of a horseshoe shaped outer ring (or) and a circular inner ring (ir), but without any indication of a fungal penetration pore.

This is evidenced through the artificial removal of fungal thalli from the cuticle of parasitized ant hosts for all four investigated Laboulbeniales (Figs. 3D, E, S1) and through histological sections of ants parasitized with *Laboulbenia formicarum* and *Rickia wasmannii* (Figs. 3A - C). That penetration structures do exist but were missed in the present study is unlikely for the following reasons. First, even minute penetration structures should have left traces of a penetration pore under dislodged fungal thalli. Instead only superficial, horseshoe shaped imprints were visible, that resembled the hoof-like foot of the thalli. Imprints occur frequently on cuticles beneath dislodged germ tubes and appressoria of many arthropod pathogenic fungi and are believed to be remnants of fungus secreted mucilage that help the fungi to attach to the cuticle of hosts (Boucias & Pendland 1991). Second, serial semithin sections with a thickness of 1 µm of the various body parts of ants were prepared for light microscopy. This ensured that potential penetration structures can be discovered irrespective of the plain of sections through the cuticle and irrespective of the plain of penetration structures in the cuticle. Third, the reported sizes of penetration structures in other Laboulbeniales range from 0.1 µm to 6 µm (see also Tab. 1). This is well within the range we could detect with our approach (section thickness 0.07 µm for TEM, 1 µm for LM).

Our result challenges the general notion that presumably all species of Laboulbeniales produce at least simple and minute penetration structures to derive resources from hosts (Scheloske 1969, Benjamin 1971) and raises questions on how and what nutrients might be gained by non-penetrating Laboulbeniales on ant hosts. Apart from direct contact with living tissues via penetration structures, it has been proposed that Laboulbeniales might absorb nutrients through the hosts' cuticle (Thaxter 1896). Insects are completely enveloped by their cuticle with the exception of tiny apertures above chemosensory cells and the pores at the tips of canals for the extrusion of wax. However, the cuticle is part of the metabolic pool and not an inert body armour and thus transport of resources to its surface is thinkable (Locke 2001). Absorbance of nutrients through the cuticle could also explain the successful staining of Laboulbeniales parasitizing carabid beetles by placing freshly excised integument on a Nile blue staining solution (Scheloske 1969).

Another hypothesis advanced to explain nutrition of non-penetrating Laboulbeniales is the use of resources available on the cuticle (CAVARA 1899, PICARD 1908). A complex of components from plants, substrate, host fecal material or from microbial flora can frequently be detected on the cuticle of insects (BOUCIAS & PENDLAND 1991) and quickly accumulates if not regularly cleaned (BÖRÖCZKY & al. 2013). On the other hand, in ants, secretions from numerous exocrine glands might provide needed resources (BILLEN 2009). Spread of these resources might be through grooming behaviours frequently seen in ants. Interestingly, it has been found that *Lasius neglectus* parasitized by *Laboulbenia formicarum* and *Myrmica scabrinodis* by *Rickia wasmannii* increase the frequency of grooming upon infection (CSATA & al. 2014, KONRAD & al. 2015). Whether this is a form of parasite manipulation to gain needed resources or simply up-regulated hygienic behaviour upon infection remains to be determined.

Nutritional resources might also be derived from the environment by Laboulbeniales. Although spore transmission of Laboulbeniales is mainly achieved by direct contact between related hosts (DE KESEL 1993, 1995), there is evidence that Laboulbeniales are capable of shifting to unrelated hosts as long as they share the same habitat (DE KESEL & HAELEWATERS 2014). Experimental infections of typical and atypical hosts showed that, although the host is essential, successful establishment of Laboulbeniales was not fully accounted for by the host physiology or cuticular characteristics, but also dependent upon the environment under which the hosts were reared (DE KESEL 1996). The importance of the environment as nutritional resource is also highlighted by the fact that *Laboulbenia ecitonis* BLUM, 1924 parasitizing army ants also can additionally infect a histerid beetle and a mite living within the ant colony (BLUM 1924). Whether the environment as nutritional resource is equally important for penetrating and non-penetrating Laboulbeniales remains to be investigated. Attempts to grow Laboulbeniales on artificial media and on exuviae of hosts (RICHARDS & SMITH 1954, 1955, 1956, WHISLER 1968) have so far been unsuccessful and limited to only a few Laboulbeniales that produce penetration structures.

Overall our study did not find penetration structures in Laboulbeniales parasitizing ant hosts. Thus recently reported fitness impacts of Laboulbeniales parasitizing *Lasius neglectus* and *Myrmica scabrinodis* ant hosts (CSATA & al. 2014, KONRAD & al. 2015), are not linked to an intrusion into the body cavity. Future comparative studies on a range of hosts involving different Laboulbeniales with and without penetration structures will have to establish the validity of this finding for Laboulbeniales in general. A better understanding of host-parasite interactions involving Laboulbeniales on ant hosts is necessary given that (I) Laboulbeniales impact their ant hosts fitness, (II) recent monitoring studies revealed that infections with Laboulbeniales are much more common among ants in Europe than previously thought (TARTALLY & al. 2007, BEZDĚČKA & BEZDĚČKOVÁ 2011a, b, LAPEVA-GJONOVA & SANTAMARIA 2011, HAELEWATERS 2012, BÁTHORI & al. 2014, 2015b), and (III) parasites have the ability to modify competition of ant communities (FEENER 2000).

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