Ann. Zool. Fennici 61: 465–478
Helsinki 19 November 2024
ISSN 0003-455X • eISSN 1797-2450

Dance of the Cave Bear: Honouring the Scientific Legacy of Björn Kurtén

Insect-mediated spore dispersal in calicioid fungi: an experimental approach

Jouko Rikkinen[1,2,*], Luka Nierhoff[3], Christina Beimforde[3] & Alexander R. Schmidt[3]

[1)] *Museum of Natural History, P.O. Box 7, FI-00014 University of Helsinki, Finland (*corresponding author's e-mail: jouko.rikkinen@helsinki.fi)*
[2)] *Organismal and Evolutionary Biology Research Programme, Faculty of Biological and Environmental Sciences, P.O. Box 65, FI-00014 University of Helsinki, Finland*
[3)] *Department of Geobiology, University of Göttingen, Goldschmidtstraße 3, DE-37077 Göttingen, Germany*

Received 4 Mar. 2024, final version received 20 May 2024, accepted 20 May 2024

Rikkinen, J., Nierhoff, L., Beimforde, C. & Schmidt, A. R. 2024: Insect-mediated spore dispersal in calicioid fungi: an experimental approach. — *Ann. Zool. Fennici* 61: 465–478.

Many calicioid fungi accumulate ascospores into an adhesive mass, called the mazaedium, at tips of stipitate apothecia. Fossil specimens from European ambers demonstrate that this morphology had evolved by the Paleogene and has since remained unchanged. The conserved maintenance of a spore-saving strategy is probably linked to animal-vectored dispersal, but experimental evidence confirming this is lacking. Here, we approached the question with a series of experiments, in which ascomata of three distantly related calicioid species were exposed to living individuals of four insect species. The results confirmed that calicioid ascospores are readily attached to any insects that touch the mazaedial spore mass. Adhered ascospores could be recovered from insect surfaces with ultrasonic cleaning. We found no significant differences in the amounts of attached ascospores of different fungal species. We discuss the new findings in the context of previous observations supporting the ecological and evolutionary role of animal-vectored dispersal in calicioid fungi.

Introduction

Calicioid fungi represent a heterogeneous assemblage of phylogenetically unrelated ascomycetes, which typically produce an adhesive, yet powdery mass of mature ascospores, called the mazaedium, on the upper surface of apothecial disks (Tibell 1984, 1999). This fascinating group of fungi includes lichen-symbiotic, parasitic and saprophytic taxa, many with stalked, pin-like ascomata. Typical lichen-forming taxa include species of *Calicium* and *Chaenotheca* (Fig. 1A–B), and *Sclerophora*. Ancestral state reconstructions by Prieto *et al.* (2013) demonstrated that the mazaedia of extant calicioid fungi evolved independently from non-mazaediate ancestors more than ten times during the diversification of inoperculate ascomycetes. The evolution typically involved the loss of existing apical ascus structures present in the ancestor of each group, and once acquired, the mazaedial character state has hardly ever been lost.

Edited by Oscar E. Wilson, University of Helsinki

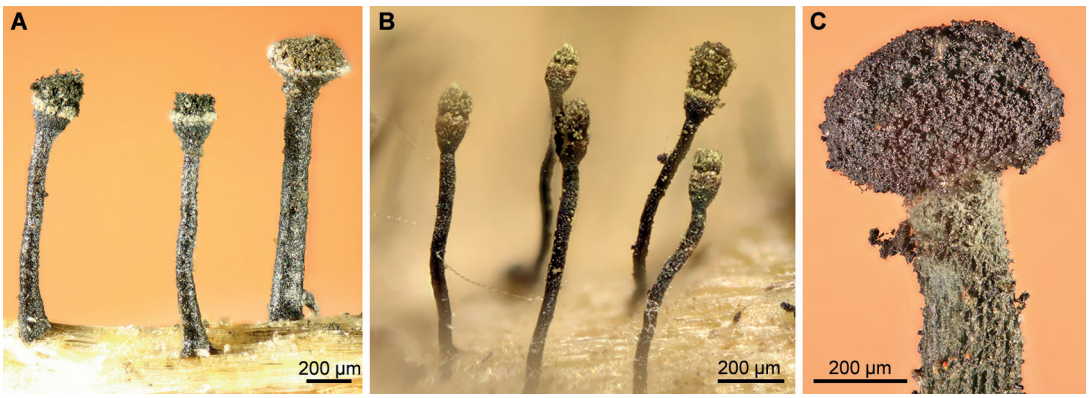

Fig. 1. Extant calicioid fungi representing three different lineages within Ascomycota. — **A**: *Calcium glaucellum* (Lecanoromycetes). — **B**: *Chaenotheca olivaceorufa* (Coniocybomycetes). — **C**: *Chaenothecopsis tsugae* (Eurotiomycetes).

Non-lichenized species such as *Chaenothecopsis* (Fig. 1C) and other members of the Mycocaliciales have maintained the apical ascus structure and forceful spore discharge but can still accumulate mature ascospores on the upper surface of apothecia, typically due to a delayed release of discharged ascospores through small openings in a thickened epithecium (Rikkinen 2003c, Tuovila *et al.* 2014). Remarkably, several lichen-parasitic *Chaenothecopsis* species are specific parasites of lichenized hosts within the genus *Chaenotheca*, and produce their pin-like ascomata on thalli and sometimes even on senescent ascomata of their hosts (Tibell 1999, Titov 2006). There are also other groups of non-lichenized ascomycetes that produce mazaedia-like spore masses but are not included in the calicioid fungi as traditionally circumscribed. For example, *Caliciopsis* and related genera in the Coryneliales have bitunicate asci where the outer wall disintegrates and mature ascospores finally accumulate as a dry mass at the ascoma beak (Benny *et al.* 1985a, 1985b, 1985c, Johnston & Minter 1989, Garrido-Benavent & Pérez-Ortega 2015, Balocchi *et al.* 2022).

As mazaedia-like spore masses occur in several distantly related ascomycete lineages and are produced in many structurally different types of ascomata, this character state must have been beneficial, and its current distribution reflects widespread convergence and parallel evolution. Indeed, Prieto *et al.* (2013) concluded that the repeated gains of the mazaedium structure within the ascomycetes must reflect some type of evolutionary advantage. Many calicioid ascospores possess a rough surface ornamentation achieved through different ontogenetic processes in different calicioid genera as first summarized by Tibell (1984). Rough spore ornamentation promotes the internal cohesion of mazaedia but may also help to adhere mature ascospores to invertebrates and other dispersal agents (Tibell 1994, Rikkinen 1995, Prieto *et al.* 2013).

While direct or experimental evidence of animal dispersal of calicioid fungi is lacking, several lines of evidence point towards its potential importance. A majority of known calicioid species live in temperate and boreal forests, especially on bark or hard lignum on basal tree trunks, and epiphytic communities rich in calicioids tend to have similar species compositions over vast geographical areas. On the other hand, some calicioids are narrow substrate specialists that grow only on specific substrates like conifer resin, angiosperm exudates, polypore fruiting bodies, or thin hardwood twigs (Rikkinen & Schmidt 2018). Many calicioids prefer mature and old-growth forests where they share specific microhabitats with wood-boring insects and other invertebrates. Wood-boring insects trigger the production of resin and other exudates which serve as a substrate for many non-lichenized calicioids (Beimforde *et al*. 2017a, 2017b). Many beetles, hymenopterans and lepidopterans are strong fliers that could transport calicioid ascospores over long distances directly into

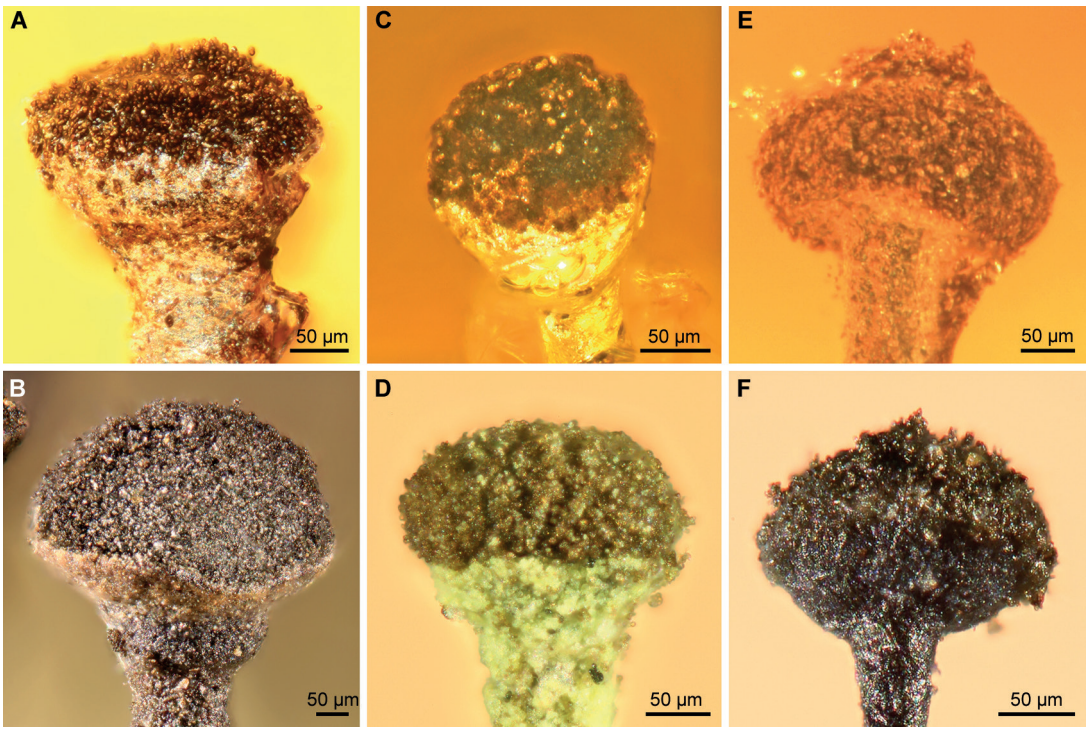

Fig. 2. Capitulum and spore mass of fossil (upper images) and extant (lower images) specimens of calicioid fungi. — **A**: Fossil *Calicium* sp. from Baltic amber (Geoscientific Collections of the University of Göttingen, GZG.BST.27296). — **B**: Extant *Calcium viride* (Lecanoromycetes). — **C**: Fossil *Chaenotheca* sp. from Baltic amber (GZG.BST.27297). — **D**: Extant *Chaenotheca* sp. (Coniocybomycetes). — **E**: Fossil *Chaenothecopsis* cf. *bitterfeldensis* from Baltic amber (GZG.BST.27286). — **F**: Extant *Chaenothecopsis proliferatus* (Eurotiomycetes). For detailed description of fossil specimens illustrated here *see* Rikkinen *et al.* (2018).

new suitable microhabitats (Biedermann & Vega 2020, Chaudhary *et al*. 2022). Woodpeckers, tree creepers and other birds may also be important for the dispersal of many calicioid species, especially those that frequently grow on lignum or resin in deep scars left by woodpeckers (Rikkinen 1995, 2003a, Elliott *et al*. 2019, Johansson *et al.* 2021).

Fossils of lichens and microfungi were long thought to be extremely rare. However, recent discoveries have shown that European Paleogene ambers are a rich source of previously largely neglected fossil lichens and microfungi (Schmidt *et al.* 2014, Hartl *et al.* 2015, Kaasalainen *et al.* 2015, Kettunen *et al.* 2015, 2016, 2017, 2018). Resinicolous calicioids typically colonize fresh exudate and produce their ascomata on semi-solid or hardened resin and on resin-impregnated wood and bark. Furthermore, many calicioid lichens frequently grow in or around resin-producing cankers and some species can also produce their ascomata on old and hardened resin. Because the ascomata of these fungi are relatively tiny, they can be easily engulfed by subsequent flows of fresh exudate and have the potential to become fossilized if the resin turns into amber. As a result, the overall prospects for finding fossil calicioids in amber are favourable. Indeed, many specimens of both resinicolous and lichen-forming calicioids have already been found from Paleogene European ambers (Rikkinen & Poinar 2000, Rikkinen 2003b, Tuovila *et al.* 2013, Sukholmlyn *et al.* 2021).

Recently, we analysed all available amber fossils of calicioid fungi to assess the applicability of the fossils for dating molecular phylogenies (Rikkinen *et al.* 2018). Many fossils are extremely well preserved and allow detailed comparison with modern taxa (Fig. 2). All specimens are confidently assigned to modern genera

and SEM investigation revealed that even fine details of the ascospore wall ultrastructure correspond to those of extant taxa. Three *Calicium* and *Chaenotheca* fossils were assigned to specific intrageneric lineages within their genera, and the numerous *Chaenothecopsis* fossils provided information of intraspecific variation within their lineage (Rikkinen *et al.* 2018).

The fossil calicioids found from Paleogene ambers almost certainly occupied microhabitats comparable to those of their extant relatives, and given what is known about the specialized ecological requirements of modern taxa, Rikkinen and Schmidt (2018) proposed a likely ecology for each amber fossil, leading to an idealized model of a forest environment where all the fossil taxa could have existed side by side. These findings supported the perception of European Paleogene amber forests as being structurally variable mosaics of dense and open forest stands (Kaasalainen *et al*. 2017, Sadowski *et al*. 2017). In these forest environments, the production of almost identical ascomata by several lineages of phylogenetically unrelated fungi has been interpreted to reflect convergent evolution towards a spore-saving strategy important for animal dispersal (Rikkinen & Schmidt 2018, Rikkinen *et al*. 2018). The apparent morphological stasis demonstrates that the adaptive morphology had evolved by the Paleogene and has since remained unchanged, i.e., a functional guild of forest fungi has conservatively maintained morphological features that are instrumental for long-term maintenance of their specialized niche.

While previous studies underlined the potential importance of animal dispersal in the ecology and evolution of calicioid fungi, no experimental evidence of this is yet available. Collecting relevant evidence of insects crawling over calicioids and detecting animal-mediated transport of ascospores is exceedingly difficult to do in the field. Therefore, here we set out to acquire new information on the potential of insect dispersal with a series of laboratory experiments, where ascomata of three distantly related calicioid species were exposed to living individuals of four insect species. The aim of this study was to determine whether fungal ascospores could become attached to living insects, and if so, to explore possible systematic differences in this respect among different fungal taxa and different insect vectors, and which morphological features of calicioid ascospores are associated with such differences.

Material and methods

Biological material and imaging

The following three calicioid species, each representing different fungal orders, were used for the experiment: *Calicium salicinum* (Caliciales, Lecanoromycetes), *Chaenothecopsis schefflerae* (Mycocaliciales, Eurotiomycetes), and *Sclerophora peronella* (Coniocybales, Coniocybomycetes) (Fig. 3). Abundant collections of each species were made in the field, that of *C. schefflerae* in New Zealand and those of the other two species in Oregon, USA.

Microscopic images of the fungi were captured with a Canon EOS 70D digital camera attached to a Carl Zeiss AxioScope A1 compound microscope. All images represent digitally stacked photomicrographs obtained from up to 125 focal layers that were merged using the software package HeliconFocus 6.3.3. A Zeiss micrometre scale was used to measure insect size and for creating the scale bars. The experimental design was documented with a Canon EOS 60D digital camera. For SEM investigation of ascospore ornamentation, spores were transferred to a carbon-covered SEM mount, coated with platinum/palladium (7 nm coat thickness) and examined under a Carl Zeiss Gemini 460 field emission scanning electron microscope.

Two species of beetles cultivated at the Department of Forest Zoology and Forest Conservation of the University of Göttingen were used in the experiments. They were *Phaedon cochleariae* (Chrysomelidae, Coleoptera; Fig. 4D), and *Tribolium castaneum* (Tenebrionidae, Coleoptera). In addition, specimens of the beetle species *Bitoma crenata* (Colydiidae, Coleoptera; Fig. 4C), and the ant species *Lasius fuliginosus* (Formicinae, Hymenoptera; Fig. 4B), were collected from the Experimental Botanical Garden of Göttingen University for the experiments.

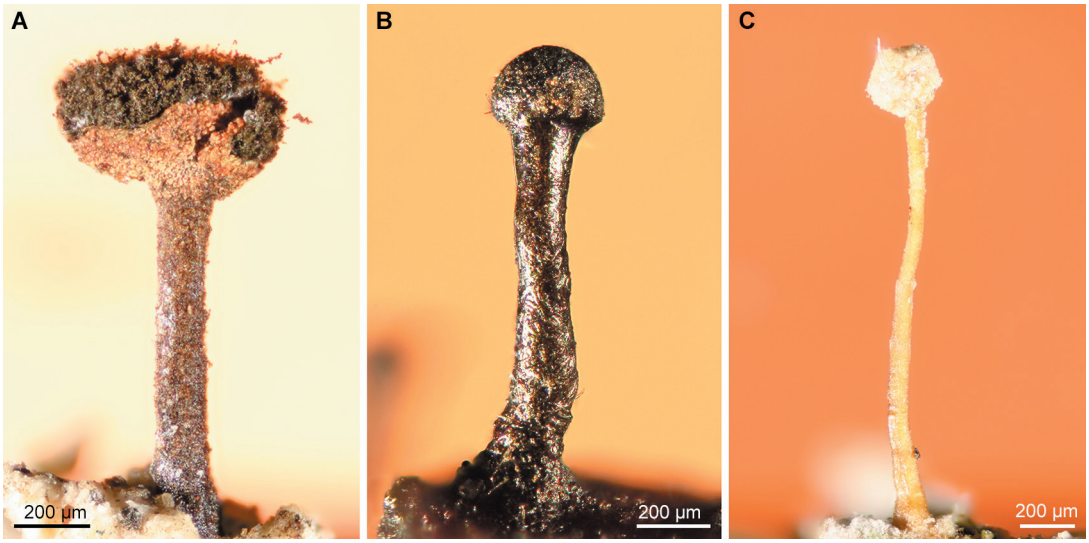

Fig. 3. Ascomata of the three species of calicioid fungi used in the experiments. — **A**: *Calicium salicinum*. — **B**: *Chaenothecopsis schefflerae*. — **C**: *Sclerophora peronella*.

Experimental setup

To determine whether calicioid ascospores attach to insects, we exposed ascomata of three different calicioid fungi to living individuals of four insect species. Clear plastic boxes measuring 10 × 7 × 4 cm (length × width × height) were divided into two compartments with cardboard cross-walls with a small rectangular opening in the middle part (Fig. 4A and B). Insects placed into one end of the box could only pass from one compartment to another through the opening in the dividing wall, and the edges of the cardboard wall were folded in to guide insects to the opening (Fig. 4B). The bottom of the box was lined with paper, as preliminary experiments had shown that the plastic was too slippery for small beetles to walk on. The opening in the cardboard wall was 5 mm high and 10 mm wide in experiments with the larger insect species and 5 mm wide in those with smaller species.

Ascomata of each fungal species with sufficiently long stipes and intact mazaedia were individually picked under a Carl Zeiss Stemi 508 dissecting microscope using sharp-tipped tweezers. The ascomata were attached upright on 20 × 20 mm glass cover slips with an epoxy two-component adhesive (Epoxy-Minute Adhesive, Weicon) (Fig. 4E–H). In each plastic box, a glass cover slip with upright fungal ascomata was placed right behind the opening in a way that it was impossible for the insects to pass through without touching the ascomata (Fig. 4B). Thus, if an insect was on the other side of the wall it meant that it had been in contact with fungal ascomata. Nine ascomata of each fungal species were arranged in three rows 1 mm apart in experiments with smaller insects, and 2.5 mm apart in experiments with larger species.

Three cover slips were prepared using ascomata of each of the three fungal species (Fig. 4E–G). Additionally, one cover slip (Fig. 4H) with five ascomata of *Chaenothecopsis schefflerae* and four ascomata of *Calicium salicinum* was prepared to test the potential simultaneous dispersal of more than one fungus species by a single insect. As controls, blank samples without ascomata were also prepared and included in the experiment. There were four boxes for each insect species plus four for control, thus 20 in total.

At the beginning of each experiment, individuals of *Tribolium castaneum*, *Bitoma crenata*, *Phaedon cochleariae* and *Lasius fuliginosus* (mean body lengths 3.47, 2.87, 3.92 and 4.85 mm, respectively) were placed into the first compartment of each box (for insect numbers *see* Table 1), with the second compartment directed towards the daylight.

Fig. 4. Experimental setup. — **A**: Example of experimental chamber. — **B**: Experimental chamber with *Lasius fuliginosus* and *Chaenothecopsis schefflerae*. — **C**: *Bitoma crenata*. — **D**: *Phaedon cochleariae*. — **E**–**H**: Cover slips with attached ascomata of calicioid fungi; (**E**) *Calicium salicinum*, (**F**) *Chaenothecopsis schefflerae*, (**G**) *Sclerophora peronella*, (**H**) mix of *Calicium salicinum* and *Chaenothecopsis schefflerae*.

The insect activity was observed to make sure the insects were able to pass through the opening, did not distinctly avoid touching the ascomata, and did not experience difficulties in

climbing over the ascomata, but we did not count how many times the insects passed through the openings. After 24 hours, all insects were collected from the box for the removal and counting of fungal spores.

Spore counts

After the experiment, all insects from each box were collected into 1.5 ml Eppendorf tubes filled with pure ethanol. The tubes were shaken in a VWR ultrasonic cleaner for 3 min to release the spores from the insects' surface. After taking the insects out with tweezers, their body length was measured under a Carl Zeiss Stemi 508 dissecting microscope. The Eppendorf tubes were then centrifuged for three minutes in an Eppendorf Centrifuge 5415 R. The upper 1.45 ml of ethanol was skimmed carefully with a pipette and the residual alcohol was pipetted onto a concave microscope slide. It was not possible to directly use ordinary cover slips because of the lack of surface tension in ethanol. Within some minutes the ethanol had evaporated and was replaced by water and the suspension with the spores was capped with a 20 × 20 mm cover slip.

The procedure was repeated for each *Phaedon cochleariae* and *Tribolium castaneum* sample, and the blank control samples. The counting of spores was initially slow because spores and other solid material washed from the insects was suspended within the liquid medium between the concave microscope slide and the cover slip. Thus later, the procedure was modified by evaporating the ethanol already from the Eppendorf tubes. The rate of evaporation was accelerated by placing the opened tubes in an Eppendorf Thermomixer at 70 °C for 10 min. The solid contents were then re-suspended in 0.05 ml distilled water, transferred onto an ordinary microscope slide and covered with a 20 × 20 mm cover slip. This modified procedure was performed for *Bitoma crenata* and *Lasius fuliginosus*, and the associated control samples.

Table 1. Numbers of ascospores of *Calicium salicinum*, *Sclerophora peronella* and *Chaenothecopsis schefflerae* recovered from individuals of *Tribolium castaneum*, *Phaedon cochleariae*, *Bitoma crenata* and *Lasius fuliginosus* placed in experimental chambers with calicioid ascomata. Insect number = total number of insects placed in the experimental chambers; Ascomata number = total number of fungal ascomata in the experimental chambers; Spore number = total number of ascospores recovered from the insects; Av. Sp/As = average number of spores recovered per fungal ascoma; Av. Sp/In = average number of spores recovered per individual insect.

		Tribolium	*Bitoma*	*Phaedon*	*Lasius*	Total
Calicium	Insect number	7	6	6	6	25
	Ascomata number	13	13	13	13	52
	Spore number	379	788	1263	3366	5796
	Av. Sp/As	29	61	97	259	111
	Av. Sp/In	54	131	211	561	232
Sclerophora	Insects number	4	3	3	3	13
	Ascomata number	9	9	9	9	36
	Spore number	339	36	324	125	824
	Av. Sp/As	38	4	36	14	23
	Av. Sp/In	85	12	108	42	63
Chaenothecopsis	Insect number	8	6	6	6	26
	Ascomata number	14	14	14	14	56
	Spore number	37	177	1072	130	1416
	Av. Sp/As	3	13	77	9	25
	Av. Sp/In	5	30	179	22	54
Total	Insect number	19	15	15	15	64
	Ascomata number	36	36	36	36	144
	Spore number	755	1001	2659	3621	8036
	Av. Sp/As	21	28	74	101	56
	Av. Sp/In	40	67	177	241	126

All 20 slides were studied under a Carl Zeiss AxioScope A1 compound microscope equipped with a Canon EOS 5D digital camera. Spores were counted manually by screening the whole area under the cover slips in rows at a 200× or 160× magnification. Ascospores of *Sclerophora peronella* were only distinguishable when using differential interference contrast and at least 200× magnification, while the darker and larger ascospores of *Calicium salicinum* and *Chaenothecopsis schefflerae* were already visible at a 160× magnification using bright light. From the mixed sample of *Calicium salicinum* and *Chaenothecopsis schefflerae*, the ascospores of each species were counted separately. The blank samples were examined to assure the insects had not brought in any foreign calicioid spores.

Data analyses

For evaluation of the insect spore dispersal, the number of spores per insect was determined. Because the spores from all insects per box were collected on a single slide and counted together, the number of spores per slide was divided by the number of insects in the respective box, giving the average number of spores detached from one individual insect. Finally, this was divided by the number of ascomata of each fungus in the respective box (usually 9, but 4 for *Calicium salicinum* and 5 for *Chaenothecopsis schefflerae* in the mixed specimen). These spore counts per insect and ascoma were compared across the four insect species, as well as among the three calicioid species. Before statistical testing, we assessed the normality of residuals and the homogeneity of variances for both groups using Shapiro-Wilk's test and Levene's test. The spore counts for the insect species met the assumptions of normality and homogeneity of variances, allowing us to use a one-way ANOVA to compare the group means. In contrast, the spore counts for the calicioid species showed heterogeneity of variances, and the spore counts for *Chaenothecopsis schefflerae* were also not normally distributed. Thus, we employed a non-parametric Kruskal-Wallis test to assess differences among the spores counts of the calicioid species. All statistical analyses were conducted using R ver. 4.3.3.

Results

Size and ornamentation of ascospores

The size, structure and ontogeny of ascospores of the three calicioid species agreed well with those reported in Tibell (1984, 1987, 1999). In *Calicium salicinum*, the ellipsoid, two-celled ascospores were (3.2)4.5–6.6(6.8) × (2.3)2.6–3.2(3.4) µm, dark greyish brown (appear black *en masse* in the mazaedium) and had a distinct ornamentation of helically arranged ridges and occasional cracks (Fig. 5A). The ascospores were often released before maturity and were thus smaller and smoother in young mazaedia. During maturation a secondary wall developed beneath the primary spore wall. The secondary wall gradually thickened until it formed the bulk of the spore wall, with the deeply ruptured primary wall forming the outer surface.

In *Chaenothecopsis schefflerae*, the ellipsoid, two-celled ascospores were (5.2)5.5–7.6(8.0) × (2.1)2.4–3.9(4.3) µm, olive green, (appear dark grey *en masse* in the mazaedium) and had a distinct ornamentation of low and uneven ridges consisting of small warts, reflecting irregularities in the outermost layer of the primary wall (Fig. 5B).

In *Sclerophora peronella*, the globose, unicellular ascospores were (3.9)4.2–5.2(5.5) µm in diameter, hyaline (appear pale brown *en masse* in the mazaedium) and had a coarse and irregular surface ornamentation (Fig. 5C). In this species, the spores were often released from asci well before maturity and thus tended to be smaller and more smooth-walled in young mazaedia. During maturation the primary wall of the ascospore increased in thickness until its outer surface was ruptured by irregular cracks.

Adherence of ascospores to living insects

In the experiments, ascospores of all three calicioid species invariably became attached to the surfaces of all insects that came into contact with the fungal ascomata. The spores of *Calicium salicinum* (mean = 35.1 spores per insect and ascoma) seemed to be more easily attached and carried than those of *Chaenothecopsis schefflerae*

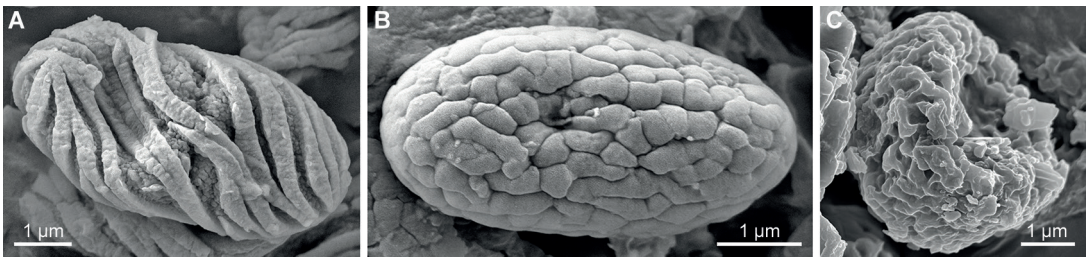

Fig. 5. Shape and ornamentation of ascospores of the three species of calicioid fungi used in the experiments. — **A**: *Calicium salicinum*. — **B**: *Chaenothecopsis schefflerae*. — **C**: *Sclerophora peronella*.

(mean = 7.09 spores per insect and ascoma) and *Sclerophora peronella* (mean = 6.84 spores per insect and ascoma). However, the Kruskal-Wallis test revealed no significant differences in the spore counts between the fungal species (H_2 = 3.186, p = 0.203) (Table 1). In the experiments involving mixtures of two calicioid species, ascospores of both were always recovered from the insects. The controls confirmed that no calicioid spores were brought into the experimental chambers from the environment. Individuals of *Bitoma crenata* and *Lasius fuliginosus*, which had been collected from the field, occasionally brought in fungal conidia, but these were easy to tell apart from the calicioid ascospores.

Individuals of *Lasius fuliginosus* (mean = 33.8 spores per insect and ascoma) seemed to carry more spores than the other species, *Phaedon cochleariae* (mean = 19.8 spores per insect and ascoma), *Bitoma crenata* (mean = 14.3 spores per insect and ascoma) and *Tribolium castaneum* (mean = 5.01 spores per insect and ascoma). However, ANOVA revealed no significant differences in spore numbers among the insect species ($F_{3,16}$ = 0.95, p = 0.44) (Table 1). None of the insect species appeared to avoid the ascomata, nor did they seem to be specifically attracted by the fungal fruiting bodies. Individuals of *Lasius fuliginosus* were quite active, passing through the opening in both directions, and soon exploring the whole chamber. Individuals of *Bitoma crenata* moved around less and often tried to get under the sheet of paper covering the chamber floor. Individuals of *Tribolium castaneum* were more active than *Bitoma crenata*, but both species experienced some difficulty in passing through the openings as most fungal ascomata were higher than the insects in question. Some ascomata were even broken off when individuals of *Tribolium castaneum* forced their way through the openings. Individuals of *Phaedon cochleariae* were quite active, frequently coming into contact with the ascomata. This was the only insect species that appeared to be attracted by light, though some individuals returned to the first compartment after first passing through the opening.

Discussion

Our results confirm that mature ascospores of calicioid fungi are easily attached to small invertebrates whenever the animals come in contact with mazaedial spore masses. All individuals of all insect species used in our experiments became carriers of ascospores and in many cases the number of ascospores attached to individual insects was in the hundreds. *Lasius fuliginosus* seemed to carry more fungal spores than the other insect species, which may be related to the larger body size and more active behaviour of this species. Conversely, the smallest and least active insect species, *Tribolium castaneum*, appeared to carry relatively few spores. The differences in spore counts among the insect species were, however, not significant. Ascospores of *Calicium salicinum* were easily attached to insects, likely due to the relatively large mazaedia of this fungus and its large ascospores with a very coarse surface ornamentation.

The conditions within the experimental chambers were of course far from those experienced by fungi and insects in their natural environments. However, the experiments clearly demonstrate that calicioid ascospores can easily

be attached to moving insects and this holds true across different calicioid lineages and different insect taxa. Several previous studies suggested that such links also exist in the wild. *Chaenothecopsis khayensis* grows on resin exuding from African mahoganies (*Khaya* sp., Meliaceae) damaged by the larvae of mahogany shoot borers (*Hypsipyla* sp.), and is likely dispersed by the adults of this moth species (Tuovila *et al*. 2011). *Chaenothecopsis perforata* grows on exudate on insect-damaged branches of *Rhus chinensis* (Anacardiaceae), and often shares its substrate with *Calciopsis rhoina*, another potentially insect-vectored fungus (Rikkinen 2000, Tuovila *et al*. 2004). The same *Chaenothecopsis* species was recently also found on three *Rhus* species native to North America, where it grows on exudate outpourings caused by insects and/or mechanical damage (Gockman *et al*. 2019). *Chaenothecopsis schefflerae* grows on branches and stems of *Pseudopanax* species (Araliaceae), again on exudates that exude from cankers caused by wood-boring insects (Beimforde *et al*. 2017b, Rikkinen & Schmidt 2018).

Resinogalea humboldtensis grows in New Caledonia on the resin of *Araucaria humboldtensis* (Araucariaceae) (Rikkinen *et al*. 2016). As the fungus has only been found from resin flows caused by invertebrates, it may depend on wood-boring insects for both nutrition and dispersal. Beimforde *et al*. (2017a) revealed that the resin flows are induced by two beetle species, both boring into branches and branchlets of the conifer. The larger beetle represents a weevil of the tribe Araucariini, an archaic lineage within the Cossoninae, accomodating the neotropical genus *Araucarius*, along with several Oceanian genera. The close association between these weevils and *Araucaria* species led us to propose that, as resin flows of araucarian conifers are known to have existed for tens of millions of years (Stockey 1994, Nohra *et al*. 2015), they may have allowed the evolution of highly specialized associations between wood-boring insects, resinicolous fungi and associated organisms.

Interestingly, Balocchi *et al*. (2023) recently described two new *Resinogalea* species from Chile, and based on sequence data, transfered the genus into Cryptocaliciales. Both new fungi were found on resin exuding from severe cankers on the branches and stems of *Araucaria araucana*, caused by the recently described *Pewenomyces kutranfy* (Coryneliaceae). These authors were able to inoculate *Araucaria* branches with isolates of the *Pewenomyces* species, causing copious resin exudation covering the point of inoculation and eventually inducing cankers similar to those seen in the field (Balocchi *et al*. 2021). Balocchi *et al*. (2021) did not comment on potential vectors of the canker-causing fungus, but noted that a low incidence of branches infested by insects and by secondary leaf pathogens were consistently observed at all field sites, and that most fungal infections took place between the end of winter and the beginning of spring. The typical spore-saving strategy, involving the production of mazaedium-like spore masses at tips of stipitate ascomata, suggests that insects or other animals may facilitate the dispersal of both *P. kutranfy* and *Resinogalea araucana*. The second *Resinogalea* species, *Resinogalea tapulicola*, is so far only known from culture. Adding further complexity to this interesting assemblage, three additional *Pewenomyces* species have since been described by Balocchi *et al*. (2022) from the same host conifer.

Recently, Beimforde *et al*. (2023) described three new resinicolous *Chaenothecopsis* species from exudates of endemic New Zealand Podocarpaceae, particularly *Prumnopitys taxifolia*. Many specimens of these resinicolous fungi had insect faecal pellets between the ascomata, with some of these consisting almost entirely of *Chaenothecopsis* spores. This suggests that associated insects had fed on the resinicolous fungi and defecated undigested ascospores. In some cases, primordia of fungal ascomata were observed growing out from faecal pellets.

Brunneocarpos banksiae (Mycocaliciales) was described growing among florets within the dense, cone-like inflorescences of *Banksia attenuata* in Australia (Crous *et al*. 2016). As the host plant is mainly pollinated by nectariphagous birds and mammals, especially by the honey possum (*Tarsipes rostratus*), one may suspect that fungal ascospores are regularly co-dispersed with pollen grains of the host (Wiens *et al*. 1979, Ritchie *et al*. 2021).

A remarkable example of marsupial-vectored dispersal is *Chaenothecopsis quitralis*, which has so far only been found growing on the viscin coats of *Tristerix corymbosus* (Loranthaceae) seeds that have passed through the intestinal track of the monito del monte (*Dromiciops gliroides*) (Messuti *et al.* 2012). A related fungus, *Chaenothecopsis viscinicola*, is only known from viscin of *Tristerix longibracteatus*. No specific information exists on the seed dispersal of the host, but birds of the families Contingidae, Tyrannidae, and Turdidae, are likely vectors of this parasitic plant (Amico *et al*. 2007, Lamilla *et al*. 2020), potentially also co-dispersing the spores of *Chaenothecopsis viscinicola*.

Symbiotic associations between fungi and insects typically involve an exchange of services, which can be grouped into several types relative to their benefits to one or both partners. These include nutrition, protection, and dispersal, and often such benefits are not exclusive. Biedermann and Vega (2020) noted that the more specialized and difficult-to-access an ecological niche is, the more benefit fungi tend to derive from assisted dispersal of their propagules. They also concluded that many insect-fungal mutualisms were probably first initiated by insects accidently dispersing fungi, which only later evolved structural adaptations for external or internal dispersal by the animal vector. This may also be the case with calicioid fungi.

In many microhabitats, several calicioid fungi regularly occur together and form characteristic communities sometimes consisting of up to ten or more different taxa (Rikkinen 1995, 2003a). Such patterns of co-occurrence are non-random and partly explained by shared substrate preferences and similar microclimatic optima. However, distantly related fungi may also have co-adapted to utilize the same dispersal vectors, thus helping to generate a specific community structure and to enhance morphological convergence and evolutionary stasis in apothecial morphology. In this context, it is relevant to note that many calicioid species produce coelomycetous and/or hyphomycetous anamorphs (Honegger 1985, Tibell 1990, 1991, 1995, 1997, Tibell & Constantinescu 1991, Beimforde *et al*. 2017b, Ertz *et al*. 2023). The conidia produced by anamorphs can potentially function not only as spermatia in sexual reproduction, but also as diaspores. One convincing example of apparent adaptation to shared animal vectors are the *Asterophoma* anamorphs of parasitic or parasymbiotic *Chaenothecopsis* species (Fig. 6), which typically develop on the thalli, ascomata and spore masses of lichenized *Chaenotheca* species (Hawksworth 1981, Tibell 1993).

Fig. 6. *Asterophoma*-anamorphs of *Chaenothecopsis viridireagens* developing on stipes of senescent apothecia of *Chaenotheca* sp.

Conclusions

While we here provided compelling new evidence of the importance of animal-vectored dispersal in calicioid fungi, several other types of ecological interactions remain unexplored. Scattered observations of animals feeding on resinicolous calicioid fungi and the fact that their ascospores can sometimes retain their viability after indigestion point towards the possible role of nutritional mutualisms between such fungi and their animal vectors. These mutualisms should be subject to further study, both through observations in the field and in controlled laboratory experiments. The experimental setup that

we introduce here could represent a useful starting point from which these questions can be explored in the future.

Acknowledgments

We thank Gerrit Holighaus (Göttingen) for providing beetle specimens from the cultures of the Department of Forest Zoology and Forest Conservation and Vincent Perrichot (Rennes) for help in the identification of field-collected insects. Andreas Kronz (Göttingen) kindly processed the ascospore samples at the Field emission scanning electron microscope. We are grateful for the statistical support provided by Katrin Mareike Meyer (Göttingen) and Daniel Gonzáles Marx (Göttingen). We thank two anonymous reviewers for their helpful comments and Oscar Wilson (Helsinki) for thoughtfully handling the manuscript. This study was supported by funds provided by the Academy of Finland (project 343113).

References

Amico, G. C., Vidal-Russell, R. & Nickrent, D. L. 2007: Relationships and ecological speciation influence of *Tristerix* (Loranthaceae): the influence of pollinators, dispersers, and host. — *American Journal of Botany* 94: 558–567.

Balocchi, F., Wingfield, M. J., Ahumada, R. & Barnes, I. 2021: *Pewenomyces kutranfy* gen. nov. et sp. nov. causal agent of an important canker disease on *Araucaria araucana* in Chile. — *Plant Pathology* 70: 1243–1259.

Balocchi, F., Barnes, I., Wingfield, M. J., Ahumada, R. & Visagie, C. M. 2023: New *Resinogalea* species from *Araucaria araucana* resin in Chile and reclassification of the genus in the Cryptocaliciomycetidae. — *IMA Fungus* 14, 16, https://doi.org/10.1186/s43008-023-00122-9.

Balocchi, F., Marincowitz, S., Wingfield, M. J., Ahumada, R. & Barnes, I. 2022: Three new species of *Pewenomyces* (Coryneliaceae) from *Araucaria araucana* in Chile. — *Mycological Progress* 21, 92, https://doi.org/10.1007/s11557-022-01840-x.

Beimforde, C., Schmidt, A. R., Tuovila, H., Kaulfuss, U., Germer, J., Lee, W. & Rikkinen, J. 2022: *Chaenothecopsis* (Mycocaliciales, Ascomycota) from exudates of endemic New Zealand Podocarpaceae. — *MycoKeys* 95: 101–129.

Beimforde, C., Seyfullah, L. J., Perrichot, V., Schmidt, K., Rikkinen, J. & Schmidt, A. R. 2017a: Resin exudation and resinicolous communities on *Araucaria humboldtensis* in New Caledonia. — *Arthropod–Plant Interactions* 11: 495–505.

Beimforde, C., Tuovila, H., Schmidt, A. R., Lee, W. G., Gube, M. & Rikkinen, J. 2017b: *Chaenothecopsis schefflerae* (Ascomycota: Mycocaliciales): a widespread fungus on semi-hardened exudates of endemic New Zealand Araliaceae. — *New Zealand Journal of Botany* 55: 387–406.

Benny, G. L., Samuelson, D. A. & Kimbrough, J. W. 1985a: Studies on the Coryneliales. II. Taxa Parasitic on Podocarpaceae: *Corynelia*. — *Botanical Gazette* 146: 238–251.

Benny, G. L., Samuelson, D. A. & Kimbrough, J. W. 1985b: Studies on the Coryneliales. III. Taxa parasitic on Podocarpaceae: *Lagenulopsis* and *Tripospora*. — *Botanical Gazette* 146: 431–436.

Benny, G. L., Samuelson, D. A. & Kimbrough, J. W. 1985c: Studies on the Coryneliales. IV. *Caliciopsis, Coryneliopsis,* and *Coryneliospora*. — *Botanical Gazette* 146: 437–448.

Biedermann, P. H. W. & Vega, F. E. 2020: Ecology and evolution of insect–fungus mutualisms. — *Annual Review of Entomology* 65: 431–455.

Chaudhary, V. B., Aguilar-Trigueros, C. A, Mansour, I. & Rillig, M. C. 2022: Fungal dispersal across spatial scales. — *Annual Review of Ecology, Evolution, and Systematics* 53: 69–85.

Crous, P. W., Wingfield, M. J., Richardson, D. M., Le Roux, J. J., Strasberg, D., Edwards, J., Roets, F., Hubka, V., Taylor, P. W. J., Heykoop, M., Martín, M. P., Moreno, G., Sutton, D. A., Wiederhold, N. P., Barnes, C. W., Carlavilla, J. R., Gené, J., Giraldo, A., Guarnaccia, V., Juarro, J., Hernández-Restrepo, M., Kolařík, M., Manjón, J. L., Pascoe, I. G., Popov, E. S., Sandoval-Denis, M., Woudenberg, J. H. C., Acharya, K., Alexandrova, A. V., Alvarado, P., Barbosa, R. N., Baseia, I. G., Blanchette, R. A., Boekhout, T., Burgess, T. I., Cano-Lira, J. F., Čmoková, A., Dimitrov, R. A., Dyakov, M. Y., Dueñas, M., Dutta, A. K., Esteve-Raventós, F., Fedosova, A. G., Fournier, J., Gamboa, P., Gouliamova, D. E., Grebenec, T., Groenewald, M., Hanse, B., Hardy, G. E. St. J., Held, B. W., Jurjević, Ž., Kaewgrajang, T., Latha, K. P. D., Lombard, L., Luangsaard, J. J., Lysková, P., Mallátova, N., Manimohan, P., Miller, A. N., Mirabolfathy, M., Morozova, O. V., Obodai, M., Oliveira, N. T., Ordóñez, M. E., Otto, E. C., Paloi, S., Peterson, S. W., Phosri, C., Roux, J., Salazar, W. A., Sánchez, A., Sarria, G. A., Shin, H.-D., Silva, B. D. B., Silva, G. A., Smith, M. Th., Souza-Motta, C. M., Stchigel, A. M., Stoilova-Disheva, M. M., Sulzbacher, M. A., Telleria, M. T., Toapanta, C., Traba, J. M., Valezuela-Lopez, N., Watling, R. & Groenewald, J. Z. 2016: Fungal planet description sheets: 400–468. — *Persoonia* 36: 316–458, https://doi.org/10.3767/003158516X692185.

Elliott, T. F., Jusino, M. A., Trappe, J. M., Lepp, H., Ballard, G.-A., Bruhl, J. J. & Vernes, K. 2019: A global review of the ecological significance of symbiotic associations between birds and fungi. — *Fungal Diversity* 98: 161–194.

Ertz, D., Diederich, P., Lendemer, J., Komposch, H., Harris, R. C. & Huereca, A. 2023: A remarkable and widespread new lichenicolous species of *Mycocalicium* (Sphinctrinaceae) producing campylidia-like conidiomata and appendiculate conidia. — *Plant and Fungal Systematics* 68: 411–423.

Garrido-Benavent, I. & Pérez-Ortega, S. 2015: Unravelling the diversity of European *Caliciopsis* (Coryneliaceae,

Ascomycota): *Caliciopsis valentina* sp. nov. and *C. beckhausii* comb. nov., with a worldwide key to *Caliciopsis*. — *Mycological Progress* 14, 10, https://doi.org/10.1007/s11557-015-1034-2

Gockman, O., Selva, S. B. & McMullin, R. T. 2019: The first report of *Chaenothecopsis perforata* from North America. — *Opuscula Philolichenum* 18: 52–57.

Hartl, C., Schmidt, A. R., Heinrichs, J., Seyfullah, L. J., Schäfer, N., Gröhn, C., Rikkinen, J. & Kaasalainen, U. 2015: Lichen preservation in amber: morphology, ultrastructure, chemofossils, and taphonomic alteration. — *Fossil Record* 18: 127–135.

Hawksworth, D. L. 1981: The lichenicolous Coleomycetes. — *Bulletin of the British Museum for Natural History* 9: 1–98.

Honegger, R. 1985: The hyphomycetous anamorph of *Coniocybe furfuracea*. — *Lichenologist* 17: 273–279.

Johnston, P. R. & Minter, D. W. 1989: Structure and taxonomic significance of the ascus in the Coryneliaceae. — *Mycological Research* 92: 422–430.

Johansson, N. R., Kaasalainen, U. & Rikkinen, J. 2021: Woodpeckers can act as dispersal vectors for fungi, plants, and microorganisms. — *Ecology and Evolution* 11: 7154–7163.

Kaasalainen, U., Schmidt, A. R. & Rikkinen, J. 2017: Diversity and ecological adaptations in Palaeogene lichens. — *Nature Plants* 3, 17049, https://doi.org/10.1038/nplants.2017.49.

Kaasalainen, U., Heinrichs, J., Krings, M., Myllys, L., Grabenhorst, H., Rikkinen, J. & Schmidt, A. R. 2015: Alectorioid morphologies in Paleogene lichens: new evidence and re-evaluation of the fossil *Alectoria succini* Mägdefrau. — *PLoS ONE* 10(6), e0129526, https://doi.org/10.1371/journal.pone.0129526.

Kettunen, E., Grabenhorst, H., Gröhn, C., Dörfelt, H., Rikkinen, J. & Schmidt, A. R. 2015: The enigmatic hyphomycete *Torula sensu* Caspary revisited. — *Review of Palaeobotany and Palynology* 219: 183–193.

Kettunen, E., Sadowski, E.-M., Seyfullah, L. J., Dörfelt, H., Rikkinen, J. & Schmidt, A. R. 2017: Caspary's fungi from Baltic amber: historic specimens and new evidence. — *Papers in Palaeontology* 5: 365–389.

Kettunen, E., Schmidt, A. R., Diederich, P., Grabenhorst, H. & Rikkinen, J. 2016: Lichen-associated fungi from Paleogene amber. — *New Phytologist* 209: 896–898.

Kettunen, E. J., Schmidt, A. R., Diederich, P., Grabenhorst, H. & Rikkinen, J. 2018: Diversity of lichen-associated filamentous fungi preserved in European Paleogene amber. — *Earth and Environmental Science Transactions of the Royal Society of Edinburgh* 107: 311–320.

Lamilla, L. A., Robayo, C. A., Castano, F., Marquínez, X. & Raz, L. 2020: Floral anatomy of *Tristerix longebracteatus* (Loranthaceae). — *Revista de Biología Tropical* 68: 87–97.

Messuti, M. I., Vidal-Russell, R., Amico, G. C. & Lorenzo, L. E. 2012: *Chaenothecopsis quintralis*, a new species of calicioid fungus. — *Mycologia* 104: 1222–1228.

Nohra, Y. A., Perrichot, V., Jeanneau, L., Le Pollès, L. & Azar, D. 2015: Chemical characterization and botanical origin of French ambers. — *Journal of Natural Products* 78: 1284–1293.

Prieto, M., Baloch, E., Tehler, A. & Wedin, M. 2013: Mazaedium evolution in the Ascomycota (Fungi) and the classification of mazaediate groups of formerly unclear relationship. — *Cladistics* 29: 296–308.

Rikkinen, J. 1995: What's behind the pretty colours? A study on the photobiology of lichens. — *Bryobrothera* 4: 1–239.

Rikkinen, J. 2000: Two new species of *Caliciopsis* (Coryneliaceae) from Hunan Province, China. — *Karstenia* 40: 147–151.

Rikkinen, J. 2003a: Calicioid lichens and fungi in the forests and woodlands of western Oregon. — *Acta Botanica Fennica* 175: 1–41.

Rikkinen, J. 2003b: Calicioid lichens from European Tertiary amber. — *Mycologia* 95: 1032–1036.

Rikkinen, J. 2003c: *Chaenothecopsis nigripunctata*, a remarkable new species of resinicolous Mycocaliciaceae from western North America. — *Mycologia* 95: 98–103.

Rikkinen, J. & Poinar, G. O. Jr. 2000: A new species of resinicolous *Chaenothecopsis* (Mycocaliciaceae, Ascomycota) from 20 million year old Bitterfeld amber, with remarks on the biology of resinicolous fungi. — *Mycological Research* 104: 7–15.

Rikkinen, J. & Schmidt, A. R. 2018: Morphological convergence in forest microfungi provides a proxy for Paleogene forest structure. — In: Krings, M., Harper, C. J., Cuneo, N. R. & Rothwell, G. W. (eds.), *Transformative Paleobotany*: 527–549. Academic Press, London.

Ritchie, A. L., Elliott, C. P., Sinclair, E. A. & Krauss, S. L. 2021: Restored and remnant *Banksia* woodlands elicit different foraging behavior in avian pollinators. — *Ecology and Evolution* 11: 11774–11785.

Rikkinen, J., Beimforde, C., Seyfullah, L., Perrichot, V. Schmidt, K. & Schmidt, A. R. 2016: *Resinogalea humboldtensis* gen. et sp. nov., a new resinicolous fungus from New Caledonia, placed in Bruceomycetaceae, a new family of Ascomycota. — *Annales Botanici Fennici* 53: 205–215.

Rikkinen, J., Meinke, K., Grabenhorst, H., Gröhn, C., Kobbert, M., Wunderlich, J. & Schmidt, A. R. 2018: Calicioid lichens and fungi in amber: Tracing extant lineages back to the Paleogene. — *Geobios* 51: 469–479.

Sadowshi, E.-M., Schmidt, A. R., Seyfullah, L. J. & Kunzmann, L. 2017: Conifers of the 'Baltic amber forest' and their palaeoecological significance. — *Stapfia* 106: 1–73.

Schmidt, A. R., Beimforde, C., Seyfullah, L. J., Wege, S. E., Dörfelt, H., Girard, V., Grabenhorst, H., Gube, M., Heinrichs, J., Nel, A., Nel, P., Perrichot, V., Reitner, J. & Rikkinen, J. 2014: Amber fossils of sooty moulds. — *Review of Palaeobotany and Palynology* 200: 53–64.

Stockey, R. A. 1994: Mesozoic Araucariaceae: an evolutionary perspective. — *Journal of Plant Research* 107: 493–502.

Sukhomlyn, M. M., Heluta, V. P., Perkovsky, E. E. Ignatov, M. S. & Vasilenko, D. V. 2021: First record of fungus of the family Mycocaliciaceae in Rovno amber (Ukraine). — *Paleontological Journal* 55: 684–690.

Tibell, L. 1984: A reappraisal of the taxonomy of Caliciales. — *Beihefte zur Nova Hedwigia* 79: 597–713.

Tibell, L. 1987: Australasian Caliciales. — *Symbolae Botanicae Upsalienses* 27: 1–279.

Tibell, L. 1990: Anamorphs in *Mycocalicium albonigrum* and *M. subtile* (Caliciales). — *Nordic Journal of Botany* 10: 221–242.

Tibell, L. 1991: The *Asterophoma* anamorph of *Chaenothecopsis savonica* and its hyphomycetous synanamorph. — *Canadian Journal of Botany* 69: 2427–2433.

Tibell, L. 1993: The anamorphs of *Chaenothecopsis viridireagens*. — *Nordic Journal of Botany* 13: 331–335.

Tibell, L. 1994: Distribution patterns and dispersal strategies of Caliciales. — *Botanical Journal of the Linnean Society* 116: 159–202.

Tibell, L. 1995: The anamorph of *Chaenothecopsis debilis*. — *Mycologia* 87: 245–252.

Tibell, L. 1997: Anamorphs in mazaediate lichenized fungi and the Mycocaliciaceae ("Caliciales s. lat"). — *Symbolae Botanicae Upsalienses* 32: 291–322.

Tibell, L. 1999: Calicioid lichens and fungi. — *Nordic Lichen Flora* 1: 20–94.

Tibell, L. & Constantinescu, O. 1991: *Catenomycopsis rosea* gen. et sp. nov. (Hyphomycetes), anamorph of *Chaenothecopsis haematopus*. — *Mycological Research* 95: 556–560.

Titov, A. [Титов, А.] 2006: [*Mycocalicioid fungi (the order Mycocaliciales) of Holarctic*]. — KMK Scientific Press, Moskva. [In Russian].

Tuovila, H., Cobbinah, J. R. & Rikkinen, J. 2011: *Chaenothecopsis khayensis*, a new resinicolous calicioid fungus on African mahogany. — *Mycologia* 103: 610–615.

Tuovila, H., Davey, M. L., Yan L. H., Huhtinen, S. & Rikkinen, J. 2014: New resinicolous mycocalicioid fungi from China. — *Mycologia* 106: 989–1003.

Tuovila, H., Schmidt, A. R., Beimforde, C., Dörfelt, H., Grabenhorst, H. & Rikkinen, J. 2013: Stuck in time — a new *Chaenothecopsis* species with proliferating ascomata from *Cunninghamia* resin and its fossil ancestors in European amber. — *Fungal Diversity* 58: 199–213.

Wiens, D., Renfree, M. & Wooller, R. O. 1979: Pollen loads of honey possums (*Tarsipes spenserae*) and nonflying mammal pollination in southwestern Australia. — *Annals of the Missouri Botanical Garden* 66: 830–838.