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Full paper

Soil burrowing *Muscina angustifrons* (Diptera: Muscidae) larvae excrete spores capable of forming mycorrhizae underground

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ABSTRACT

Muscina angustifrons (Diptera: Muscidae) is a mycophagous species exploiting a variety of fungi including ectomycorrhizal fungi. It has been reported that larvae of this species feed on sporocarps including spores and full-grown larvae leave sporocarps and pupate $0-6$ cm below the soil surface. In this study, we examined whether *M. angustifrons* larvae have capacities to transport ectomycorrhizal fungal spores and enhance the formation of ectomycorrhiza on host-plant roots. In the present experiments, full-grown larvae usually moved horizontally 10 to 20 cm from feeding sites and burrowed underground. These wondering larvae retained ectomycorrhizal fungal spores in their intestines and excreted them after moving to underground pupation sites. Excreted spores retained germination and infection capacities to form ectomycorrhiza on host-plant roots. In the infection experiments, ectomycorrhizal fungal spores applied in the vicinity of underground host-plant roots were more effective to form ectomycorrhiza in comparison with those applied on the ground surface, suggesting that transportation of spores below the ground by *M. angustifrons* larvae could enhance ectomycorrhizal formation. These results suggest that *M. angustifrons* larvae act as a short-distance spore transporter of ectomycorrhizal fungi.

1. Introduction

Ectomycorrhizal fungi are usually associated with arboreal plants and exhibit a high diversity in forest ecosystems (Molina et al. 1992). As well as many other fungi, some ectomycorrhizal fungi are assumed to disperse their spores by wind (Ingold 1971; Halbwachs and Bässler 2015). In several species of ectomycorrhizal fungi, it has been reported that ectomycorrhizal formation does not occur when the density of spores is low, i.e. less than 10^3–10^4 spores per seedling (Rincón et al. 2001). However, such aggregated distribution of spores would not so frequently occur if spores are dispersed by wind. For example, Li (2005) showed in his study on *Amanita muscaria* that the average density of spores was $7137/m^2$ at a distance of 0.3 m from sporocarp and only $940/m^2$ at a distance of 5.2 m from sporocarp. On the other hand, high-density spore patches could more frequently occur if spores are carried by animals or insects (Tuno 1999; Kobayashi et al. 2017). There are two ways for animals and insects to carry fungal spores; i.e., keeping spores on their body surface or in intestines. In the latter case, animals and insects feed fungal body including spores and excrete spores with feces. High-density spore patches are expected to occur more frequently if spores are carried in intestines.

Animals and insects of various taxa have been reported to feed saprotrophic and ectomycorrhizal fungal sporocarps; e.g., monkeys (Hanson et al. 2006), deer (Ashkannejhad and Horton 2006), rodents (Fogel and Trappe 1978; Vernes and Dunn 2009; Vernes and McGrath 2009), birds (Simpson 2000), lizards (Cooper and Vernes 2011), slugs (Buller 1909; McGraw et al. 2002), flies (Hackmann and Meinander 1979; Toda et al. 1999), beetles (Crowson 1984; Newton 1984), and springtails (Sawahata et al. 2000). So far, at least some of them have been revealed to act as spore dispersers (Ingold 1971; Fogel and Trappe 1978; Malloch and Blackwell 1992; Tuno 1998, 1999; Lilleskov and Bruns 2003). For example, spores of coprophilous fungi and hypogeous ectomycorrhizal fungi are transported in intestines of herbivorous mammals (Ingold 1971; Fogel and Trappe 1978; Vernes and Dunn 2009). In addition, stinkhorns have been believed to be entomophily because they produce spores in liquid gleba with strong odor instead of releasing the spores in the air (Ingold 1971). Furthermore, spores of an ectomycorrhizal fungus, *Tomentella sublilacina,* are transported by soil invertebrates and their predators (Lilleskov and Bruns 2005).

The efficiency of animals and insects as spore dispersers is partly dependent on the degree of spore damages received in the intestines. Lim (1977) showed that *Ganoderma pseudoferreum* spores ingested by *Limonia* larvae have a capacity to germinate. In addition, Kobayashi et al. (2017) revealed in their experiments that some spores that pass through the intestines of drosophilid flies still maintain germination capacity although some are not viable. Further, Kobayashi et al. (2017) showed that colored spores were less damaged than colorless spores. The efficiency as spore dispersers of ectomycorrhizal fungi would also depend on whether animals and insects carry spores to the vicinity of underground host-plant roots. If carried by wind, spores would usually remain above-ground, and therefore their infection on host-plant roots is less likely to occur. If spores are carried underground by animals or insects, infection could more likely to occur. Such transportation may be realized by animals and insects that burrow underground.

However, there have been only a few studies on spore dispersal by burrowing animals and insects (e.g., Lilleskov and Bruns 2005).

Here we investigate whether *Muscina angustifrons* Loew (Diptera: Muscidae) larvae can act as a spore disperser of ectomycorrhizal fungi. This fly species is a dominant mycophagous insect in the Hokuriku district of Japan; its larvae feed on sporocarps of a variety of fungi including ectomycorrhizal fungi of the orders Boletales, Russulales and Agaricales (Akaishi and Nakamura 2005). In our preliminary study (Kitabayashi et al. 2016), a large amount of spores were observed in intestines of *M. angustifrons* larvae. In addition, larvae of this species burrow underground for pupation probably to avoid parasitoid attack and desiccation (Nakasuji 1965). From these results, it is assumed that *M. angustifrons* larvae are able to carry ectomycorrhizal fungal spores to the vicinity of underground host-plant roots. Here, we investigate 1) what distance *M. angustifrons* larvae move for pupation, 2) whether they carry spores below the ground or not, and 3) whether spores that pass their digestive tract retain capacities to germinate and form mycorrhiza on host-plant roots.

2. Materials and methods

2.1. Movements of larvae to pupation site
Full-grown (third instar) *M. angustifrons* larvae leave breeding sites and burrow underground for pupation (Nakasuji 1965). To assess their importance as spore dispersers, we firstly investigated what distance they move for pupation. Sporocarps of ectomycorrhizal fungi were collected from forests in and near Kanazawa (33.6 ºN, 136.6 ºE), central Japan, from July to August in 2015. Collected sporocarps were identified to species by morphology according to Ikeda (2015). After species identification, we cut sporocarps with a cutter knife in halves to check the presence of muscid larvae. When dipteran larvae were present, they were identified to family by morphological characteristics according to Aoki (1999). Since *M. angustifrons* is the only mycophagous Muscidae species in the Hokuriku district of Japan (Tuno, unpublished observation), all muscid larvae found in this study were assigned as *M. angustifrons*. Among ectomycorrhizal fungi collected in this study, three species of *Russula* (*R. alboareolata*, *R. foetens* and *R. neoemetica*: Russulaceae) and two species of Boletaceae (*Tylopilus neofelleus* and *Boletus pseudocalopus*) were infested by *M. angustifrons* larvae. For each fungal species, one sporocarp infested with muscid larvae was placed on a wet paper towel at the center of plastic containers (40 cm in width × 90 cm in length × 12 cm in height) with moistened vermiculite (6 cm in depth) on the bottom. The containers were kept at room temperature (22–25 ºC). When *M. angustifrons* larvae in the sporocarps became the last instar (i.e., 1 cm in length and 3 mm in width or larger), we removed the paper towel and placed sporocarps directly on vermiculite. Three–5 days after all larvae left sporocarps, the number of *M. angustifrons* pupae found in three subdivided areas (i.e., an area within 10 cm from the center, an area from the radius of 10 cm to 20 cm from the center, and an area outside of 20 cm from the center) were examined; i.e., vermiculite layers were collected from these three areas and sorted for *M. angustifrons* pupae. When larvae were observed to move along the walls of the containers, they were judged to have

moved over 20 cm because the nearest wall from the center located at a distance of 20 cm.

2.2. The presence/absence of spores in intestines of wondering larvae

The importance of *M. angustifrons* larvae as dispersers of mycorrhizal spores is also dependent on whether they carry spores underground or not, because mycorrhizal infection on host-plant roots usually occurs underground. To examine whether they carry spores underground or not, we carried out experiments with three *Russula* species, *Russula alboareolata R. foetens and R. neoemetica.* For each mushroom species, one sporocarp infected by *M. angustifrons* larvae was collected from forests in and near Kanazawa in July and August in 2015 and placed on vermiculite in the center of a plastic container with moistened vermiculite as mentioned above. When larvae become the last instar and started wondering or burrowing, they were collected and examined for the presence or absence of spores in their intestines; after their body surface was rinsed with sterilized distilled water to remove spores, they were dissected to observe their intestines under a microscope. Experiments were carried out at room temperature (22–25 ℃).

2.3. Germination of spores

Germination capacity of spores that passed through the digestive tract of *M. angustifrons* larvae was investigated for *Russula alboareolata*, *Russula* sp., *Gyroporus longicystidiatus* and *Boletus* sp. For each mushroom species, one sporocarp infected by *M. angustifrons* larvae was collected from forests in and near Kanazawa in August and September in 2014 and 2015, placed on paper towel spread over wet vermiculite (1 cm in thickness) in plastic cups (60 mL), and kept at 22°C under a long daylength (15 h light–9 h dark). When *M. angustifrons* larvae grew to the last instar, three individuals were randomly collected from each sporocarp. After their body surface was rinsed with sterilized distilled water to remove spores, they were placed in plastic cups (30 mL) moistened by spraying distilled water and allowed to excrete for three to five hours. Excrements were collected into plastic sampling tubes (1.5 mL) using micropipettes and stirred in sterile water. The density of spores in suspension was measured using hemocytometer and adjusted to 10^6–10^8/mL. Suspension (10 µL) with spores was dripped on 2% agar plates added with Benomyl (10 mg/L) and Chloramphenicol (100 mg/L) as antibiotics and expanded using a conradi rod. The plates were sealed and cultured at 22°C under a long day-length for two days. Spores on the culture plates were observed under a microscope (Axio Imager 2, Carl Zeiss Inc., Tokyo), and the proportion of germinated spores in one or two microscopic field(s) (3.80 mm^2 in visual field) was examined. For control, *R. alboareolata* and *G. longicystidiatus* were used; a part of cap was cut off from the above-mentioned sporocarps (i.e., those used to examine the effect of passing through the digestive tract of *M. angustifrons* larvae), and spores were collected from spore prints or directly from lamellae. The germination capacity was examined as described above. The germination rate was compared between excreted and control spores by χ^2 test.

2.4. Mycorrhizal formation of spores#

Pinus thunbergii seeds were obtained from Forest Experiment Station of Ishikawa prefecture. After the surface was sterilized in 2% (w/w) calcium hypochlorite solution for 10 min and rinsed three times with sterilized distilled water, the seeds were placed on 2% agar plates in Petri dishes (9 cm in diameter and 2 cm in height) and kept at 22°C under a long daylength for a week. We autoclaved culture bottles; vermiculite: sphagnum moss (80: 1 w/w) moistened with modified Norkrans's "C" (MNC) without glucose (ca. 35 mL medium/100 mL substrate) (Yamada and Katsuya 1995) in SM sample bottles (450 mL, Sansyo Co. Ltd., Tokyo). Aseptic seedlings (3–7 d after germination) were then individually transplanted into the autoclaved bottles in the clean bench to avoid contamination.

We collected *Russula alboareolata* (5 sporocarps), *Russula neoemetica* (4), *Suillus granulatus* (2) and *Tylopilus vinosobrunneus* (3) infected by *M. angustifrons* larvae from forests in and near Kanazawa in August and September in 2015. From sporocarp of each mushroom species, suspensions of control spores and those that passed through the digestive tract of *M. angustifrons* larvae (treated spores) were prepared as described above except that five larvae were used for the preparation of treated-spore suspension. Thus, 28 suspensions (14 for each of control and treated spores) were prepared. The density of spores in these suspensions was adjusted to 10^6–10^7/mL. The estimated number of treated spores applied to a seedling was $2.48\times10^6\pm2.11\times10^6$ (mean±SD), while the estimated number of control spores applied was $1.67\times10^6\pm0.68\times10^6$. There was no significant difference in the density of spores between the treated- and control-spore suspensions (Wilcoxon test: $Z = 0.03536$, $P > 0.5$). In this process, the number of spores excreted by one larva was estimated as 9.91×10^5; i.e., on average, 49.55×10^5 spores were estimated to occur in a suspension prepared from excretes of five larvae.

One week after transplanting aseptic seedlings into the bottles, 500 µL of the spore suspension was applied on the surface of vermiculite or 3–6 cm below the surface using a micropipette according to the previous observation that *M. angustifrons* larvae usually pupate 0–6 cm below the soil surface (Nakasuji 1965). After suspension was applied, the mouth of bottles was covered with three layers of polyvinylchloride film (Riken wrap, Riken Vinyl Industry Co. Ltd., Tokyo), on which two vents (ca. 6 mm in diameter) were made for ventilation. Each vent was covered with a fluorocarbon membrane filter (pore size: 0.5 µm) (Milliseal, Millipore Co. Ltd., Yonezawa) to avoid contamination by microorganisms. The bottles were maintained at 22°C under a long daylength in an incubator equipped with four 200 W and one 15 W fluorescent lamps (BITEC-300L, Shimazu, Co. Ltd, Japan) as light source. To one seedling, 500 µL water were additionally supplied two weeks after the planting; water was gently supplied along the wall of the culture bottle to prevent spore flowing. To other seedlings, water was not supplied additionally. After six months, plants were taken out from the bottles, rinsed with sterilized distilled water and observed for the formation of ectomycorrhizal sheaths on the root tip under a dissecting light microscope at 20 × magnification (Leica EZ4, Leica Co. Ltd., Tokyo). Tips of roots with sheaths were cut with scissors and preserved in ethanol in 1.5mL tubes at -30°C as voucher specimen. The rate of ectomycorrhizal formation was compared between excreted and

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control spores and between spores applied below the surface and those on the surface using Fisher's exact test after pooling data for all fungal species. In this study, the rate of ectomycorrhizal formation was not compared between mushroom species, because the sample size was small.

3. Results

3.1. Travel distance of wondering larvae ♯

In experiments using five fungal sporocarps of five different species of ectomycorrhizal fungi, a total of 50 *M. angustifrons* pupae (or larvae) were found; 12 (24%) in an area within 10 cm from the center, 28 (56%) in an area from the radius of 10 cm to 20 cm from the center, and 10 (20%) in an area outside of 20 cm from the center (Table 1). Among 10 individuals found in an area outside of 20 cm from the center, four (40%) were found on the wall of container, suggesting that they would move further if the wall was not present.

3.2. The presence/absence of spores in intestines of wondering larvae

In total, 20 larvae were collected; 14 on the surface of vermiculite and 6 below the surface (Table 2). Except two larvae occurring below the surface, all retained fungal spores in their intestines. The two larvae without spores had hard cuticle, suggesting that they had started pupation and already purged gut contents. These results suggest that last-instar larvae of *M. angustifrons* could transport fungal spores to their underground pupation sites.

3.3. Germination of spores

The germination rate of spores that passed through the digestive tract of *M. angustifrons* larvae was 1.6–2.8%, and that of control spores was 1.8–3.4% (Table 3). In *R. alboareolata* and *G. longicystidiatus*, the difference in the germination rate between control spores and those passed through the digestive tract was not significant (χ^2 test ; $P = 0.823$ in *R. alboareolata*, $P = 0.444$ in *G. longicystidiatus*).

3.4. Mycorrhizal formation

Twenty-eight spore suspensions prepared from 14 sporocarps (5 for *R. alboareolata*, 4 for *R. neoemetica*, 2 for *S. granulatus* and 3 for *T. vinosobrunneus*) considerably varied in amount, so that suspensions with limited amounts were applied only to single seedlings either on or below the surface and those with a lot of amounts were applied to 2-4 seedlings. Therefore, the number of seedlings used in the experiments varied to some extent by mushroom species, spore types

(treated or control) and position of application (on or below the surface) (Table 4). The occurrence of fungal sheath on root tips was confirmed in 13 *P. thunbergii* seedlings out of 44 (Table 4, Figure 1). The rate of ectomycorrhizal formation was significantly higher when spores were applied below the surface (47.8%, N=23) than when applied on the surface (9.5%, N=21) (Fisher's exact test, $P = 0.008$, N = 44), but was not significantly different between control spores (20.8%, N=24) and those passed through the digestive tract of *M. angustifrons* larvae (40.0%, N=20) (Fisher's exact test, $P > 0.05$, N = 44). A seedling supplied with only water did not form mycorrhizae.

4. Discussion

It was once thought that the major mode of proliferation of ectomycorrhizal fungi is vegetative growth, but it has been revealed in the last two decades that sexual reproduction by spores also frequently occurs (Dahlberg and Stenlid 1994; Zhou et al. 1999; Redecker et al. 2001; Lian et al. 2006; Yoshioka et al. 2012). However, how ectomycorrhizal spores infect host plants in the field is largely unknown. In this study, we focused on *M. angustifrons* larvae as an agent to carry ectomycorrhizal spores and enhance mycorrhizal formation. *M. angustifrons* larvae feed on mycelium and also other dipteran larvae in sporocarps (Akaishi and Nakamura 2014), and they retain a large number of spores in their intestines although they seem to have no or poor capacity to digest spores (Kitabayashi et al. 2016). To assess their importance in mycorrhizal formation, it is important to know when and where they excrete spores. It has been known that insect larvae excrete all intestine contents at pupation (Nation 2008). The present results indicate that *M. angustifrons* larvae retained spores in intestines even after they started wondering for pupation and excrete them with other intestine contents at the time of pupation. In addition, it appeared that fungal spores excreted by *M. angustifrons* larvae retain capacity to form ectomycorrhiza on *P. thunbergii* roots. Furthermore, ectomycorrhizal formation was more successful when spore suspension was applied in the vicinity of host-plant roots than when applied on the surface of ground (i.e., the surface of vermiculite layer in this study), although the germination rate of spores was low whether they remained untreated or passed through the digestive tract of *M. angustifrons* larvae. It is not known why the germination rate was so low, but some substances secreted from host-plant roots may be critical for the germination of ectomycorrhizal fungal spores (Fries et al. 1984; Kikuchi et al. 2007).

It has been reported that mycorrhizal formation is dependent on the spore density; i.e., 10^3–10^6 spores are needed per seedling for successful infection in so-far-studied ectomycorrhizal fungal species (Rincón et al. 2001). Such high density patches of spores would rarely occur if spores are dispersed by wind (e.g., Li 2005), but could be realized if spores are transported in insect intestines, because the number of spores found in intestines is very large as revealed in the present and previous studies (Lilleskov and Bruns 2005; Kobayashi et al. 2017). In this study, the number of spores excreted by one larva is estimated to be 10^5 to 10^7, promising densities for the ectomycorrhizal formation.

We demonstrated that the rate of ectomycorrhizal formation was significantly higher when spores were applied below the surface of vermiculite layer than when applied on the surface, in consistence with Ishida et al. (2008) results that the germination rate of ectomycorrhizal spores increases when cultured in the vicinity of host-plant roots. Ectomycorrhyzae predominantly occur at the depth of 10 cm below the ground or shallower (Ingleby et al. 1997). In this respect, *M. angustifrons* larvae could also be an efficient disperser of ectomycorrhyzal fungal spores, because they usually pupate $0-6$ cm below the soil surface (Nakasuji 1965).

Thus, *M. angustifrons* larvae could enhance the mycorrhizal formation by transporting spores to the vicinity of host-plant roots. On the other hand, wind-dispersed spores could also be transported close to underground host-plant roots by rainwater or dew (Halbwachs and Bässler 2015). However, it is still uncertain how efficiently spores were transported by water. This is one of future research subjects.

In this study, *M. angustifrons* larvae transported spores only short distances (approximately 10–20 cm from sporocarps). However, spore transportation of such short distances can be adaptive. Recent population genetic studies on ectomycorrhizal fungi have suggested that many different genotypes exist within the range of a few meters (Gherbi et al. 1999; Abesha et al. 2003). This means that the replacement of ectomycorrhizal individuals occurs at a very small spatial scale. We speculate that short-distance transportation by muscid larvae can play an important role in competition with neighboring ectomycorrhizal individuals.

Transportation of spores by *M. angustifrons* larvae or other insects may have some other benefits for fungi. For example, old sporocarps of several fungal species including ectomycorrhizal fungi sometimes retain spores on lamellae or tubes without discharging them in the air (Kobayashi et al. 2017). Insects may play an important role in dispersing these spores. In addition, transporting in their intestines, insects may protect spores from sunlight or desiccation which could markedly reduce the germination capacity of spores, especially those with thin cell-wall (Norros et al. 2015).

M. angustifrons larvae were frequently found in sporocarps of ectomycorrhizal fungi of Boletales, Russulales and Agaricales in the Hokuriku district of Japan (Akaishi and Nakamura 2014). In Europe, *Pegomyia* (Diptera: Faniidae) flies, of which larvae also burrow underground for pupation, have been reported to prefer fungi of Boletales (Hackman and Meinander 1979; Ståhls et al. 1989). Many species of Heleomyzidae (Diptera) and Mycetophilidae (Diptera) are also known to exploit fungal sporocarps and pupate in the soil, but it is not known whether larvae of these species transport fungal spores or not. We need to expand our knowledges on natural histories of these sporocarp-inhabiting fly larvae to understand their significance in spore dispersion of ectomycorrhizal fungi.

In this study, there are some points to be noticed. First, the ectomycorrhizal formation rate was not compared between different fungal species due to the small number of replication. Second, we did not use living larvae in the application of spores to seedlings in the mycorrhizal formation experiment. We may have missed some important effects of living larvae. Third, the timing of spore excretion by larvae was studied in laboratory. In fields, they may excrete in a

different timing, but it is almost impossible to observe larval excretion of spores in fields. If we can demonstrate that the presence of larvae has positive effects on ectomycorrhizal formation in natural environments, the importance of *M. angustifrons* larvae in the spore dispersal will be more strongly indicated.

We reported for the first time that *M. angustifrons* larvae could act as a short-distance spore transporter of ectomycorrhizal fungi; they transport spores to rhizosphere of host plants by burrowing underground for pupation. Some fungi have been thought to be entomophily or animal-borne, because they do not have structure to discharge spores into the air. On the other hand, fungi that can discharge spores into the air do not always discharge all spores into the air (Kobayashi et al. 2017). Such undischarged spores may be transported by insects or animals, but it is difficult to prove that these spores are actually transported by insects or animals. Our study revealed that spores of some ectomycorrhizal fungi could be transported by fly larvae. Further comparative study is needed to evaluate which of transportation by wind or fly larvae has a larger contribution to the production of the next generation.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country where they were performed.

REFERENCES

Abesha E, Caetano AG, Høiland K, 2003. Population genetics and spatial structure of the fairy ring fungus *Marasmius oreades* in a Norwegian sand dune ecosystem, *Mycologia* 95: 1021–1031; http://dx.doi.org/10.1080/15572536.2004.11833018.

Akaishi D, Nakamura K, 2005. Seasonal occurrence and food resources of *Muscina angustifrons* (Loew) in a temperate broad-leaved secondary forest in Kanazawa, Japan. *Medical Entomology and Zoology* 56: 135–137; http://doi.org/10.7601/mez.56.135.

Akaishi D, Nakamura K, 2014. Omnivorous and predaceous larval habits of *Muscina angustifrons* (Loew)(Diptera, Muscidae). *Medical Entomology and Zoology* 65: 195–199; http://doi.org/10.7601/mez.65.195.

Aoki J, 1991 *Pictorial Keys to Soil Animals of Japan.* Tokai University Press, Japan.

Ashkannejhad S, Horton TR, 2006. Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving *Pinus contorta*, suilloid fungi and deer. *New Phytologist* 169: 345–354; http://dx.doi.org/10.1111/j.1469-8137.2005.01593.x.

Buller AHR, 1909. The dispersion of spores by animals-co-prophilous hymenomycetes slugs and hymenomycetes. In: *Researches on Fungi.* Longmans, Green and Company, London, pp 224–232.

Cooper T, Vernes K, 2011. Mycophagy in the larger bodied skinks of the genera *Tiliqua* and *Egernia*: are there implications for ecosystem health? *Australian Zoologist* 35: 681–684; https://doi.org/10.7882/AZ.2011.020.

Crowson RA, 1984. The associations of Coleoptera with Ascomycetes In: Wheeler QD, Blackwell M (eds.), *Fungus-insect relationships: perspectives in ecology and evolution.* Columbia University Press, New York, pp 256–285.

Dahlberg A, Stenlid J, 1994. Size, distribution and biomass of genets in populations of *Suillus bovinus* (L.: Fr.) Roussel revealed by somatic incompatibility. *New Phytologist* 128: 225–234; https://doi.org/10.1111/j.1469-8137.1994.tb04006.x.

Fogel R, Trappe JM, 1978. Fungus consumption (mycophagy) by small animals. *Northwest Science* 52: 1–31.

Fries N, 1984. Spore germination in the higher Basidiomycetes. *Plant Sciences* 93: 205–222; http://doi.org/10.1007/BF03053077.

Gherbi H, Delaruelle C, Selosse M, Martin F, 1999. High genetic diversity in a population of the ectomycorrhizal basidiomycete laccaria amethystina in a 150-year-old beech forest. *Molecular Ecology* 8: 2003–2013; http://doi.org/10.1046/j.1365-294x.1999.00801.x

Hackman W, Meinander M, 1979. Diptera feeding as larvae on macrofungi in Finland. *Annales Zoologici Fennici* 16: 50–83.

Halbwachs H, Bässler C, 2015. Gone with the wind–a review on basidiospores of lamellate agarics. *Mycosphere* 6: 78–112; http://doi.org/10.5943/mycosphere/6/1/10.

Hanson AM, Hall MB, Porter LM, Lintzenich B, 2006. Composition and nutritional characteristics of fungi consumed by *Callimico goeldii* in Pando, Bolivia. *International Journal of Primatology* 27: 323–346; 10.1007/s10764-005-9014-z.

Ikeda Y, 2015. *New edition: Hokurikuno-kinoko-zukan*. Hashimoto Kakubundo, Japan.

Ingleby K, Diagne O, Deans J, Lindley D, Neyra M, Ducousso M, 1997. Distribution of roots, arbuscular mycorrhizal colonisation and spores around fast-growing tree species in Senegal. *Forest Ecology and Management* 90: 19–27; http://dx.doi.org/10.1016/S0378-1127(96)03875-3.

Ingold CT, 1971. *Fungal spores. Their liberation and dispersal*. Clarendon Press, Oxford.

Ishida TA, Nara K, Tanaka M, Kinoshita A, Hogetsu T, 2008. Germination and infectivity of ectomycorrhizal fungal spores in relation to their ecological traits during primary succession. *New Phytologist* 180: 491–500; http://dx.doi.org/10.1111/j.1469-8137.2008.02572.x.

Kikuchi K, Matsushita N, Suzuki K, Hougetsu T, 2007. Flavonoids induce germination of basidiospores of the ectomycorrhizal fungus *Suillus bovinus. Mycorrhiza* 17: 563–570;

http://dx.doi.org/10.1007/s00572-007-0131-8.

Kitabayashi K, Tuno N, Hosaka K, Yaguchi Y, 2016. Natures of ingested basidio-spores in dipteran larvae inhabiting sporophores of Agaricomycetidae (in Japanese with English summary). *Japanese Journal of Mycology* 57: 69–76.

Kobayashi M, Kitabayashi K, Tuno N, 2017. Spore dissemination by mycophagous adult drosophilids, *Ecological Research* 32: 621-626. doi:10.1007/s11284-017-1477-9.

Li DW, 2005. Release and dispersal of basidiospores from *Amanita muscaria* var. *alba* and their infiltration into a residence. *Mycological Research* 109: 1235–1242; http://dx.doi.org/10.1017/S0953756205003953.

Lian C, Narimatsu M, Nara K, Hogetsu T, 2006. *Tricholoma matsutake* in a natural *Pinus densiflora* forest: correspondence between above-and below-ground genets, association with multiple host trees and alteration of existing ectomycorrhizal communities. *New Phytologist* 171: 825–836; http://dx.doi.org/10.1111/j.1469-8137.2006.01801.x.

Lilleskov EA, Bruns TD, 2003. Root colonization dynamics of two ectomycorrhizal fungi of contrasting life history strategies are mediated by addition of organic nutrient patches. *New Phytologist* 159: 141–151; http://dx.doi.org/10.1046/j.0028-646x.2003.00794.x.

Lilleskov EA, Bruns TD, 2005. Spore dispersal of a resupinate ectomycorrhizal fungus, *Tomentella sublilacina*, via soil food webs. *Mycologia* 97: 762–769; http://dx.doi.org/10.1080/15572536.2006.11832767.

Lim TM, 1977. Production, germination and dispersal of basidiospores of *Ganoderma pseudoferreum* on Hevea. *Journal of the Rubber Research Institute of Malaysia* 25: 93–99.

Malloch D, Blackwell M, 1992. Dispersal of fungal diaspores. In: Carroll GC, Wicklow DT (eds.), *The fungal community: its organization and role in the ecosystem*. Marcel Dekker Inc., New York, pp 147–171.

McGraw R, Duncan N, Cazares E, 2002. Fungi and other items consumed by the blue-gray taildropper slug (*Prophysaon coeruleum*) and the papillose taildropper slug (*Prophysaon dubium*). *The Veliger* 45: 762–769;

Molina R, Massicotte H, Trappe JM, 1992. Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. In: Allen MF (eds.), *Mycorrhizal functioning: an integrative plant-fungal process*. Chapman & Hall, London, pp 357–423.

Nakasuji F, 1965. Defference of the activity of hymenopterous parasites with special reference to the depth of the pupation site of fly larvae (in Japanese). *Konchū* 33: 441–445.

Nation JL, 2008. *Insect Physilogy and Biochemistry, second edition*, Boca Raton.

Newton AF, 1984. Mycophagy in Staphylinoidea (Coleoptera). In: Wheeler QD, Blackwell M (eds.), *Fungus–Insect Relationships, Perspectives in Ecology and Evolution*. Columbia University Press, New York, pp 302–323.

Norros V, Karhu E, Nordén J, Vähätalo AV, Ovaskainen Ot, 2015. Spore sensitivity to sunlight and freezing can restrict dispersal in wood-decay fungi. *Ecology and Evolution.* 5: 3312–3326; http://dx.doi.org/doi: 10.1002/ece3.1589.

Redecker D, Szaro TM, Bowman RJ, Bruns TD, 2001. Small genets of *Lactarius xanthogalactus*,

Russula cremoricolor and *Amanita francheti* in late-stage ectomycorrhizal successions. *Molecular Ecology* 10: 1025–1034; http://dx.doi.org/10.1046/j.1365-294X.2001.01230.x.

Rincón A, Alvarez IF, Pera J, 2001. Inoculation of containerized *Pinus pinea* L. seedlings with seven ectomycorrhizal fungi. *Mycorrhiza* 11: 265–271; http://dx.doi.org/10.1007/s005720100127.

Sawahata T, Soma K, Ohmasa M, 2000. Number and food habit of springtails on wild mushrooms of three species of Agaricales. *Edaphologia* 66: 21–34.

Simpson J, 2000. More on mycophagous birds. *Australasian mycologist* 19: 49–51.

Ståhls G, Ribeiro E, Hanski I, 1989. Fungivorous *Pegomya* flies: spatial and temporal variation in a guild of competitors. *Annales Zoologici Fennici* 26: 103–112.

Toda MJ, Kimura MT, Tuno N, 1999. Coexistence mechanisms of mycophagous drosophilids on multispecies fungal hosts: aggregation and resource partitioning. *Journal of Animal Ecology* 68: 794–803; http://dx.doi.org/10.1046/j.1365-2656.1999.00328.x.

Tuno N, 1998. Spore dispersal of *Dictyophora* fungi (Phallaceae) by flies. *Ecological Research* 13: 7–15; http://dx.doi.org/10.1046/j.1440-1703.1998.00241.x.

Tuno N, 1999. Insect feeding on spores of a bracket fungus, *Elfvingia applanata* (Pers.) Karst.(Ganodermataceae, Aphyllophorales). *Ecological Research* 14: 97–103; http://dx.doi.org/10.1046/j.1440-1703.1999.00290.x.

Vernes K, Dunn L, 2009. Mammal mycophagy and fungal spore dispersal across a steep environmental gradient in eastern Australia. *Austral Ecology* 34: 69–76; http://dx.doi.org/10.1111/j.1442-9993.2008.01883.x.

Vernes K, McGrath K, 2009. Are introduced black rats (*Rattus rattus*) a functional replacement for mycophagous native rodents in fragmented forests? *Fungal Ecology* 2: 145–148; https://doi.org/10.1016/j.funeco.2009.03.001.

Yamada A, Katsuya K, 1995. Mycorrhizal association of isolates from sporocarps and ectomycorrhizas with *Pinus densiflora* seedlings. *Mycoscience* 36: 315–323; http://dx.doi.org/10.1007/BF02268607.

Yoshioka H, Isobe K, Inui H, Kikuti J, 2012. Spatial distributin of sporocarps of ectomycorrhizal fungi in a *Quercus variabilis* - *Q. glauca stand* (in Japanese with English summary). *Bulletin of Center for Natural Environmental Education, Nara University of Education* 13: 1–13.

Zhou Z, Miwa M, Hogetsu T, 1999. Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). *New Phytologist* 144: 55–63; http://dx.doi.org/10.1046/j.1469-8137.1999.00504.x.

Figure legends

Fig. 1 – Ectomycorrhizal roots of *Pinus thunbergii* six months after application of spores of *Russula alboarealata* (A), *Russula neoemetica* (B), *Suillus granulatus* (C) and *Tylopilus vinosobrunneus* (D). Spores from feces of *Muscina angustifrons* larvae were applied 3–6 cm below the surface (A, B and D). Spores directly collected from sporocarp were applied on the surface (C).

Table 1 – Number of *Muscina angustifrons* larvae found in area within 10 cm from sporocarps, area from a radius of 10 cm to 20 cm from sporocarps and area outside of 20 cm from sporocarps.

Fungal species	Number of sporocarps	Number of larvae found		
		<10 cm	10–20 cm	>20 cm
Russula alboarealata	1	3	8	4
Russula foetens	1	3	4	1
Russula neoemetica	1	2	6	2
Tylopilus neofelleus	1	4	6	2
Boletus pseudocalopus	1	0	4	1
Total	5	12 (24%)	28 (56%)	10 (20%)

Table 2 – Number of *Muscina angustifrons* larvae with (+) or without (-) of spores in intestines. Larvae were collected from surface of vermiculite or below surface.

Fungal species	Number of sporocarps	Larvae on surface		Larvae below surface	
		+	-	+	-
Russula alboarealata	1	5	0	1	1
Russula foetens	1	4	0	1	1
Russula neoemetica	1	5	0	2	0
Total	3	14	0	4	2

Table 3 – Germination rates (%) of spores that passed through the
digestive tract of *Muscina angustifrons* (Ma) and control spores.

Fungal species	Number of sporocarps	Ma	control
Russula alboarealata	1	1.6 (2224)	1.8 (108)
Russula sp.	1	1.8 (2110)	–
Gyroporus longicystidiatus	1	2.8 (1211)	3.4 (1068)
Boletus sp.	1	2.4 (511)	–

Numbers in parentheses refer to the total number of spores observed.

Table 4 – Number of pine seedlings that formed ectomycorrhiza by spores that
passed through the digestive tract of *Muscina angustifrons* larvae (Ma)
and control spores applied on surface or 3–6 cm below surface.

Fungal species	Surface		Below surface	
	Ma	control	Ma	control
Russula alboarealata	0 (2)	0 (2)	2 (3)	1 (3)
Russula neoemetica	0 (3)	0 (3)	0 (2)	1 (3)
Suillus granulatus	—	0 (4)	3 (3)	—
Tylopilus vinosobrunneus	1 (3)	1(4)	2 (4)	2 (5)
Total	1 (8)	1 (13)	7 (12)	4 (11)

Numbers in parentheses refer to the number of pine seedlings used in experiments.