Ant’s Nest as a Microenvironment: distinct Mucoromycota (Fungi) community of the red wood ants’ (Formica polyctena) mounds

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Abstract

Many social insect species build nests, which strongly differ from the surrounding environment and are often occupied by specific organismal communities. In temperate forests, red wood ants (e.g. Formica polyctena) are known to create such distinct, highly developed nests, which consist of large, above-ground mounds. Those structures are built primarily out of plant matter collected from the forest litter. Common fungal dwellers of forest litter are representatives of Mucoromycota, engaged in the decomposition process of this substrate. However, data on co-occurrence or interactions between these ants and fungi remains unknown. In order to elucidate these interactions we characterized Mucoromycota communities of Formica polyctena nests and the surrounding forest litter. We sampled four sites, twice in a season and used: a culturomics approach, complemented with DNA barcoding to describe fungal communities; PERMANOVA test and non-metric multidimensional scaling ordinations to compare those communities; and multilevel pattern analysis to indicate taxa associated with the mounds. Our results show that the Mucoromycota community of Formica polyctena’s mound is specific and more stable than the community of the surrounding forest litter. While representatives of Entomortierella lignicola and Absidia cylindrospora clade were found to be associated with the mound environment, representatives of Umbelopsis curvata and Podila verticillata-humilis clade were associated with forest litter, and were rarely present in the mounds. Our findings strongly suggest that the red wood ants’ nest is a specific microenvironment in the temperate forest floor, which is a preferred microhabitat for the mound-associated Mucoromycota, possibly adapted to live in close proximity to ants.

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Abstract

Many social insect species build nests, which strongly differ from the surrounding environment and are often occupied by specific organismal communities. In temperate forests, red wood ants (e.g. *Formica polyctena*) are known to create such distinct, highly developed nests, which consist of large, above-ground mounds. Those structures are built primarily out of plant matter collected from the forest litter. Common fungal dwellers of forest litter are representatives of *Mucoromycota*, engaged in the decomposition process of this substrate. However, data on co-occurrence or interactions between these ants and fungi remains unknown. In order to elucidate these interactions we characterized *Mucoromycota* communities of *Formica polyctena* nests and the surrounding forest litter. We sampled four sites, twice in a season and used: a culturomics approach, complemented with DNA barcoding to describe fungal communities; PERMANOVA test and non-metric multidimensional scaling ordinations to compare those communities; and multilevel pattern analysis to indicate taxa associated with the mounds. Our results show that the *Mucoromycota* community of *Formica* polyctena’ s mound is specific and more stable than the community of the surrounding forest litter. While representatives of *Entomortierella lignicola* and *Absidia cylindrospora* clade were found to be associated with the mound environment, representatives of *Umbelopsis curvata* and *Podila verticillata-humilis* clade were associated with forest litter, and were rarely present in the mounds. Our findings strongly suggest that the red wood ants’ nest is a specific microenvironment in the temperate forest floor, which is a preferred microhabitat for the mound-associated *Mucoromycota*, possibly adapted to live in close proximity to ants.

Keywords:

ant-fungal interactions; microenvironments; nest microbiome; red wood ants; *Mucoromycota*; temperate forests;

Introduction

The majority of ant species lead a social and stationary lifestyle, which includes active nest building (Hölldobler & Wilson, 1990). While forming their nests, ants highly modify their surroundings (De Almeida et al., 2020; Folgarait, 1998; Jouquet et al., 2006; Kovář et al., 2013; Meyer et al., 2013) and thus are often perceived as ecosystem engineers (Del Toro et al., 2012; Wills & Landis, 2018). In the environment, such actively maintained nests act as distinctive, environmental islands (Boots et al., 2012; Dauber et al., 2001; Folgarait, 1998), and are occupied by specific bacterial (Boots et al., 2012; Lindström et al., 2019; Lucas et al., 2019; Song et al., 2023; Travanty et al., 2022) and animal communities (Hölldobler & Kwapich, 2022; Kronauer & Pierce, 2011; Parmentier et al., 2014; Wells et al., 2017). Some specifically adapted species, called obligate myrmecophiles, are even present exclusively in ants’ nests (Hölldobler & Kwapich, 2022; Kronauer & Pierce, 2011; Parker & Grimaldi, 2014; Parmentier et al., 2014).

In the case of fungi, the number of studies that show a presence of distinct, fungal communities in ant-made environments is still scarce (Boots et al., 2012; Brinker et al., 2019; Lindström et al., 2019; Lucas et al., 2019), with just a few examples of extensively studied mutualistic fungi present in ants’ nests. Apart from fungus-growing ants, with an extreme example of coevolution between leaf-cutting ants and *Leucoagaricus gongylophorus* (Currie, 2001; Dejean et al., 2023; Hölldobler & Wilson, 1990; Mueller et al., 1998), fungal symbionts of ant nests have also been described for some ant species occupying domatia and for cardboard nest-forming ants (Defossez et al., 2009; Nepel et al., 2014, 2016; Ruiz-González et al., 2010). In the case of ‘carton ants’, ‘black-yeasts’ were found to overgrow the walls of their nests and thus increase the strength of the whole structure (Dejean et al., 2023; Ruiz-González et al., 2010). In all those ant-fungal mutualistic interactions, while the beneficial role of fungal partners differs, from nutritional to structural (Blatrix et al., 2012; Chomicki & Remmer, 2017; Dejean et al., 2023; Hölldobler & Wilson, 1990; Ruiz-González et al., 2010), the fungal presence always relies on the abundance and type of accumulated organic matter.

Interestingly, the nest-nycobiota of the mound-building *Formica*, which creates one of the most complex and long-lasting, organic nests (Stockan & Robinson, 2016), has been severely understudied. The unique feature of their nest is a presence of an above-ground mound built mostly out of dead plant material collected from the surroundings (Czechowski et al., 2012; Scherba, 1958; Stockan & Robinson, 2016, Fig. 1). Until now, out of all
mound-building *Formica* species, only the mycobiota of *Formica exsecta* have been comprehensively described in a series of studies by Lindstrom and others (2019, 2021, 2023). Those studies show that the mycobiome of *F. exsecta* ants’ mounds is specific, more stable, more abundant, and significantly different from the mycobiome of surrounding forest soil. Additionally, representatives of *Ascomycota* (*Exophiala, Oidiodendron*, *Sclerocondidium*) and *Mucoromycota* (*Umbelopsis*) have been proposed as indicator taxa for the mound environment (Lindström et al., 2019).

However, the knowledge about the mycobiota of the red wood ants (RWA; *Formica rufa* group as defined by Borowiec et al., 2021), another well-known, monophyletic group of mound-building *Formica*, remains very limited. Representatives of this group are commonly mixed in coniferous temperate forests, strongly affecting these ecosystems (Czechowski et al., 2012; Frouz & Jilková, 2008; Jilková, 2015; Jørgensen et al., 2008; Kilpeläinen et al., 2007; Stockan & Robinson, 2016). In the forest floor, they build large, domed, and long-lasting mounds, which reach up to two meters in height and up to one cubic meter in volume (Czechowski et al., 2012; Stockan & Robinson, 2016, Fig. 1). The most common organic components of those mounds are: pine needles, little twigs, pieces of bark and other small, usually lignified plant parts, and a decaying stump located in the center (Castella et al., 2008; Czechowski et al., 2012; Frouz et al., 2016; Stockan & Robinson, 2016). Thanks to RWA’s activity, physical and biochemical properties of their mounds significantly differ from the surrounding forest litter (Domisch et al., 2009; Frouz et al., 2016; Frouz & Jilková, 2008; Jilková, 2015; Kadochová, 2017; Kilpeläinen et al., 2007). The mound is characterized by maintained, adjusted, usually lowered humidity (Frouz et al., 2016), stabilized, increased throughout most of the year temperature, oscillating between -2 and 32 centigrade degrees (Frouz et al., 2016; Frouz & Finer, 2007), slightly alkalized pH (Jilková et al., 2012), and increased amount of nutrients such as polysaccharides and simple sugars (Domisch et al., 2009; Frouz et al., 2016; Frouz & Finer, 2007; Kilpeläinen et al., 2007). The mound is additionally distinct from the litter by the abundant presence of antimicrobial substances (formic acid and coniferous resin) incorporated into the material (Brutsch et al., 2017; Brutsch & Chapuisat, 2014; Castella et al., 2008), and the usual lack of plant-growth (Frouz & Jilková, 2008; Laakso & Setälä, 1998).

Importantly, the mound itself is not a homogenous environment. The more central part of the mound, the more stable the temperature throughout the day and throughout the seasons (Frouz, 2000), with the temperature in the center of the mound not dropping below the freezing point (Frouz et al., 2016). Additionally, higher terpene and resin concentrations are noted within the interior of the mound (Brutsch & Chapuisat, 2014; Sorvari & Hartikainen, 2021). Moisture within the mound also differs, with the surface layer being usually more moist than the interior part (Elo et al., 2018). Finally, the surface layer contains plant matter of smaller sizes than the mound interior (Maavara et al., 1994).

The microbial biomass in the mounds is usually higher than in the surrounding soil (Golubev & Bab’eva, 1972; Laakso & Setälä, 1998; Maksimova et al., 2016). So far, only yeast communities of RWA’s mounds have been analyzed. Golubev and Bab’eva (1972) and Maksimova et al. (2016) have shown that yeast communities from ants’ nests differ from yeast communities of the surrounding soil. In both of these studies, representatives of *Debaryomyces* (*Debaryomyces hansenii*, *Schwanniomyces polymorphus*, and *S. vanrijiae*) were shown to be associated with ants’ nests. Moreover, differences in yeast community composition were also observed within the nest, with a higher number of yeasts and higher specificity of yeast community found deeper in the mound in comparison to the surface layer (Maksimova et al., 2016).

However, increased simple sugars concentrations noted in the RWA’s mounds, would suggest that also representatives of *Mucoromycota* (as defined in Naranjo-Ortiz and Gabaldón, 2019) often referred to as ‘sugar fungi’, should be expected in this specific environment. Similar to RWA, diverse *Mucoromycota* representatives are commonly present in soil, litter, rhizosphere and dead wood of coniferous and mixed temperate forests (Bahnmann et al., 2018; Carreiro & Koske, 1992; Gorfer et al., 2021; Grantina et al., 2012; Kwaśna et al., 2016; Osono et al., 2006; Summerbell, 2005; Tedersoo et al., 2014; Toju & Sato, 2018). They are also known to be engaged in the decomposition of coniferous needles (Millar, 2012; Osono et al., 2006), which are one of the main building materials of RWA’s mounds. In recent years, some *Mucoromycota* fungi were
isolated from RWA-associated substrates. Representatives of the insect-associated *Entomortierella* genus have been isolated from the content of infrabuccal pockets of *F. rufa* (Clark, 2002). In a study focused on the mycobiota of *F. polyctena* ants, strains of *Mucor*, *Entomortierella*, and *Absidia* were noted (Siedlecki et al., 2021). Moreover, *Mortierella formicae* has also been isolated and described from a cadaver of *F. polyctena* ant (Hyde, Norphanphoun, Abreu, Bazzicalupo, Mortimer, et al., 2017).

Knowing on one hand about the common presence of Mucoromycota (MM) in the temperate forest litter and being aware of specific, more homogenous properties of RWA’s mounds, we hypothesize the existence of a more stable, distinct MM community occurring in this ant-made environment. Further, we hypothesize the existence of symbiotic MM species preferentially or exclusively present in the mounds. To test it, we describe and compare the MM community present in mounds of *F. polyctena* (RWA) with the MM community of the surrounding forest litter. To characterize fungal communities, we used a culturomics approach complemented by isolates’ DNA barcoding. To indicate taxa associated with the mounds we performed a multilevel pattern analysis. Finally, we discuss why this specific, ant-made environment could work as a preferred microhabitat for some MM, possibly adapted to cohabit in RWA nests.

**Materials and methods**

**Study object**

The ant *Formica polyctena* is one of the most common species belonging to RWA, a sister species to *F. rufa*, an oligotope of coniferous and mixed temperate forests (Borowiec et al., 2021; Czechowski et al., 2012). Within mound-building *Formica* ants, *F. polyctena* is making the biggest polycalic colonies (up to 400 million individuals), with huge, long-lasting individual nests (over 1 million individuals) and mounds (even > 3m diameters), located usually in more shaded spots compared to other RWA (Czechowski et al., 2012; Stockan & Robinson, 2016).

**Study scheme (⇒ Figure 2)**

**Study material collection**

The collection of study material was conducted twice, on the 22nd of August and the 5th of October in 2020, in the pine forests of Mazovian Voivodeship (Poland). Material was collected from 4 different sites separated by at least 15 km. Each time, sampling at each site included a collection of three different substrates: two nest substrates (mound surface - MS and mound interior - MI) and forest litter - FL (Fig. 2). For the MS samples 25 ml of material was collected from 4 different sides of the mound and merged. For MI samples 100 ml of mound material was excavated at a depth of 15 cm from the central part of the mound. For FL samples 25 ml of litter from a forest floor was collected at four distinct spots surrounding the mound and merged. Each collection spot for the FL was located at least 10 meters away from the mound. The material was collected into sterile, plastic containers and transported on ice to the laboratory, where it was stored at -20°C until further analysis. In total, 24 samples were collected (8 FL, 8 MS, and 8 MI). Ants were identified as *Formica polyctena* before the sampling. Ant colony identification was based on the morphology of 5 workers per nest, using Czechowski and others (2012) key. Ant’s nest material was collected under the permission of The Regional Directorate for Environmental Protection (RDOŚ) in Warsaw. Permit number: WPN-I.6401.428.2020.PK.2. More metadata on sampling and sampled mounds is available in Table A of Appendix.

**Isolation of Mucoromycota**

For each collected sample, the material was firstly sifted through a 1 mm sieve and then mixed with sterile sand until final dilution of 1:199, following Warcup’s soil plate method, with a modification by Manka (1964) for topsoil, and then plated with 10 replicates. Each replicate contained 0.1 g of diluted material and was evenly distributed onto Petri dishes (90 mm) containing twice-diluted Sabouraud Dextrose Agar medium (SDA:WA, 1/1), with added chloramphenicol (25μg/ml). The inoculated plates were placed in dark conditions and incubated at 18°C for 7 days. After 7 days, all Mucoromycota colonies (MM cfu) were counted for each plate.
Identification of isolates

Only *Mucoromycota* representatives were selected for further studies. The isolates were assigned to taxonomic units based on molecular and morphological characteristics. Macroscopic features of the fungal colonies were observed using a stereoscopic microscope ("Nikon SMZ 800" and EOS camera) and strains’ micromorphology was studied on slides stained using lactophenol cotton blue under a light microscope ("Nikon ELIPSE Ni" and Nikon DS - Ri 2 camera with NIS Elements software). For sporulating *Mucoromycotina* (as defined in Naranjo-Ortiz & Gabaldón, 2019), all grown colonies sharing similar morphology were grouped into distinct morphotypes (Khalabuda, 1973; Skirgiello et al., 1979; Watanabe, 2002). For each morphotype, up to three representative strains (one per studied substrate) were preserved as axenic cultures. For Mortierellomycotina (as defined in Naranjo-Ortiz & Gabaldón, 2019) and non-sporulating *Mucoromycotina*, axenic cultures were preserved for all grown colonies.

Total genomic DNA was extracted from all preserved axenic cultures using the ExtractMe Genomic DNA Kit (Blirt S.A., Gdańsk, Poland), according to the manufacturer’s protocol. Molecular identification was based on the internal transcribed spacer region (ITS) using primer pairs ITS1f and ITS4 (White et al., 1990). For strains for which we were unable to obtain ITS sequence, the large subunit nuclear ribosomal DNA (LSU) marker was sequenced, using primers pairs: LROR (Rehner & Samuels, 1994) and LR5 (Vilgalys R & Hester M, 1990) or NL1 and NL4 (O’Donnell, 1993). The DNA of selected molecular markers was amplified using programs described in Okrasińska et al. (2021) for ITS and Siedlecki et al. (2023) for LSU. PCR products were purified with the ExtractMe DNA Clean-Up & Gel-Out kit (Blirt S.A.) and sequenced using the Sanger method by an external company, Genomed S.A. (Warsaw, Poland). Forward and reverse sequences were assembled using the DNA Subway software (Williams et al., 2014).

Obtained consensus sequences were compared with data available in NCBI GenBank (ncbi.nlm.nih.gov accessed on 28 December 2023) using the BlastN algorithm (Altschul et al., 1990). The species-level names were assigned when morphology based identification was in line with top blast hits (refined to type strains and reference strains deposited in recognized public culture collections), meeting the following three criteria: (1) sequence coverage > 90%, (2) sequence identity > 97% for ITS and > 98% for LSU, and (3) differentiation from the closest species > 0.7% for ITS and > 0.3% for LSU. Otherwise, the term “species clade” or higher level taxonomic rank was used to name isolated taxonomic units.

Statistical Analysis

Analysis and data visualization were performed using R v4.1.2 in RStudio (R Core Team, 2020; RStudio Team, 2020). The Shannon diversity index was calculated using a vegan package (Oksanen et al., 2019). Differences in Shannon diversity indexes between the study treatments were tested using ANOVA with a post-hoc Tukey test. Differences in community compositions were first tested for homogenous dispersion within and among groups (betadisper in vegan; Bray–Curtis), and then compared with permutational multivariate analysis of variance (PERMANOVA; adonis; Bray–Curtis distance, 999 permutations) with Bonferroni adjustment for all substrates, sampling sites, and collection month. Bray-Curtis dissimilarities were used to visualize differences between sites. Non-metric multidimensional scaling ordinations (Bray–Curtis distances) were used to visualize community compositions. A Wisconsin double standardization was performed to reduce the signal of abundantly grown species. Species significantly shaping the composition of *Mucoromycotina* communities (p < 0.05) displayed on the ordination plot were analyzed by the envfit (permutations = 999) function within the package vegan (Oksanen et al., 2019). In order to indicate taxa associated with a specific substrate, Multilevel pattern analysis (MPA) was conducted using the indicspecies package (Cáceres & Legendre, 2009), both correlation indices and indicator value functions were used in the analysis. Figures were generated with ggplot2 (Villanueva & Chen, 2019).

Results

In the study, we isolated 1301 *Mucoromycota* colony forming units (*Mucoromycotina* cfu) from 24 samples: 8 mound interior (MI), 8 mound surface (MS), and 8 forest litter (FL). In general, the number of isolated *Mucoromycotina* cfu did not differ significantly between studied substrates (ANOVA, p=0.68, F=0.39) and accounted on average for
Podila verticillata-humilis exclusively (98% of this taxa cfu) (Fig. 6B). On the other hand, strains of prevalent in the mound substrates (100% MS and 88% MI samples), where they were occurring almost more prevalent in the mound environments (Fig. 6A). Commonly isolated were also representatives of Absidia curvata of Mucoromycota. Our study showed that Mortierellaceae were isolated more frequently from the forest litter (88% of this taxa cfu) (Fig. 6B). Within Mortierellaceae, representatives of Entomortierella lignicola, Umbelopsis vinacea, and U. angularis as taxa associated with the mound and U. curvata as taxon associated with the forest litter (Fig. 5).

In both, correlation indices and indicator values analyses, Entomortierella lignicola was shown to be associated with the mound material, both with the surface and the interior of the mound (MPA, r = 0.69, p = 0.001; IV = 0.58, p = 0.002). Additionally, in the correlation indices analysis, Absidia cylindrospora clade was shown to be associated with the interior of the mound (MPA, r = 0.62, p = 0.001) and U. curvata with the forest litter (MPA, r = 0.47, p = 0.043). In the indicator values analysis, Podila verticillata-humilis clade (sensu Vandepol et al., 2020) turned out to be associated with forest litter (MPA, IV = 0.72, p = 0.018).

The most commonly isolated were representatives of the genus Umbelopsis. U. isabellina was the most abundant species (50% of MM cfu), which was recorded from all of the studied samples (Fig. 6A). Representatives of U. curvata were recorded more often from FL and MS samples, and representatives of U. angularis were more prevalent in the mound environments (Fig. 6A). Commonly isolated were also representatives of Absidia, with A. coerulca being as prevalent in FL as in MI samples, and A. cylindrospora being more abundant inside the nest (82% of this taxa cfu) (Fig. 6B). Within Mortierellaceae, representatives of E. lignicola were prevalent in the mound substrates (100% MS and 88% MI samples), where they were occurring almost exclusively (98% of this taxa cfu) (Fig. 6B). On the other hand, strains of Podila verticillata-humilis clade were isolated more frequently from the forest litter (88% of this taxa cfu) (Fig. 6B).

Discussion

Our study showed that Mucoromycota (MM) communities formed on the surface and in the interior of Formica polyctena’s mounds were similarly abundant and diverse as MM communities of the surrounding forest litter. However, the fungal community of the mound, especially the interior part of it, was more stable throughout the seasons (Fig. 3, 4). Even though most of the MM taxa were isolated from each of the studied substrates (Fig. 6), we observed a clear distinction between the MM communities of the mound and of the surrounding forest litter (Fig. 5). While strains of Umbelopsis isabellina were common in all studied substrates, representatives of Entomortierella lignicola and Absidia cylindrospora clade were mound-associated, and representatives of Umbelopsis curvata and Podila verticillata-humilis clade were litter-associated. Although the taxonomic composition of MM communities found in the mound interior and the mound surface was similar, representatives of the A. cylindrospora clade were abundant only in the mound interior (Fig. 6).
The presence of Mucoromycota communities in *F. polyctena*’s mounds suggests that antifungal substances used by those ants to decrease microbial growth, such as collected tree resin and produced formic acid (Brutsch et al., 2017; Brutsch & Chapuisat, 2014; Castella et al., 2008), are not enough to stop the development of MM species. Additionally, as representatives of MM are common in forest litter (Gorfer et al., 2021; Qu et al., 2021; Tedersoo et al., 2014), they are most likely transferred into the mound together with plant material collected by ants. Representatives of MM were also part of the fungal community described for the mostly organic mounds of *Formica exsecta* (Lindström et al., 2019, 2021).

The higher stability of MM communities between the seasons in the mounds than in the litter could be a result of more stable environmental conditions (especially temperature) occurring in the ant-made environment throughout the year (Frouz et al., 2016; Frouz & Finer, 2007). Our results are in line with the results of Lindström et al. (2021) study in which more stable microbial communities were observed in mounds of *F. exsecta* throughout seasons and years, strengthening the hypothesis that nests of mound-building *Formica* could act in the forest floor as a reservoir for microbial taxa less tolerant of climatic fluctuations (Lindström et al., 2021). Further studies that would include winter sampling while describing the RWA mounds’ mycobiota could help to verify this hypothesis.

Specific physical and chemical properties of *F. polyctena*’s mounds highly distinguish this ant-made microenvironment from the surrounding forest litter. These properties most likely shape the specific mounds’ Mucoromycota communities observed in our work. In previous studies specificity of fungal communities of ants’ mounds was also observed for the mounds of other mound-building *Formica* (*F. aquilonia, F. exsecta, F. rufa*, and *F. uklei*) (Duff et al., 2016; Golubev & Bab’eva, 1972; Lindström et al., 2019; Maksimova et al., 2016). Therefore, our results strengthen the mound’s ecosystemic distinctiveness hypothesis. In the case of *F. polyctena*, population stability, large size, and connectivity of mounds in polycalic colonies made by this species (Czechowski et al., 2012; Stockan & Robinson, 2016), may result in even stronger specificity of their mycobiota.

Highly overlapping mound inside and mound surface clusters in NMDS analysis (Fig. 5) suggest that despite some differences being noted between those substrates (Brutsch & Chapuisat, 2014; Elo et al., 2018; Frouz et al., 2016; Maavara et al., 1994; Sorvari & Hartikainen, 2021), they are not big enough to differentiate MM communities present in those microenvironments. On the other hand, the observed lack of significant differences in MM communities could have been a result of the lack of winter or spring sampling in our study. Especially because big differences in temperature between the surface and inside part of the mound are observed during those seasons (Frouz et al., 2016; Frouz & Finer, 2007). However, even with warmer seasons sampling, we observed a higher prevalence of litter-associated taxa (*P. verticillata/humilis* clade and *U. curvata*) in the mound surface than in the mound interior, suggesting that the mound surface could work as an ecotone between the litter and the interior of the mound (Fig. 6).

Fungi from the *Umbelopsis* genus are known as common coniferous forest litter dwellers. They are often isolated from decaying coniferous needles, roots, bark, and wood (Kaaeik & Kennebfelt, 1957; Kuhlman, 1969; Kwasna et al., 2016; Meredith, 1960; Osono et al., 2006; Sewell, 1959; Söderström, 1975), as well as as root endophytes of coniferous trees (Hoff et al., 2004; Kernaghan & Patriquin, 2011; Rim et al., 2021; Térhonen et al., 2014). The similar abundance of *Umbelopsis* representatives in the mound material as in the litter suggests that available nutrients and conditions are good enough for a stable *Umbelopsis* presence in the *Formica polyctena* nests and that resin and formic acid presence in the mound is not a barrier for these fungi. Omnispreadence of *Umbelopsis* shown in our study somewhat contradicts the findings of Lindström et al. (2019). In their study, the genus *Umbelopsis* was listed as a core taxon for the *Formica exsecta* mound environment. Possible explanations encompass different methodological approaches, as only molecular methods were used in Lindström’s study, or some unknown environmental differences between studied litter.

Even though we did not observe a strong preference towards any of the studied substrates for the whole *Umbelopsis* genus, we did observe such preferences on the species level. While strains of *U. isabellina* were commonly isolated from all substrate types, *U. curvata* occurred more often in the forest litter. As representatives of *U. isabellina* are commonly isolated from fresh or decaying wood of coniferous trees (Fisher
Species of Mortierellaceae are known to be common in temperate coniferous forest soil and litter (Qu et al., 2021; Santalahti et al., 2016; Tedersoo et al., 2014), found also within fungal communities decomposing coniferous needles (Osono et al., 2003), and wood (Behnke-Borowczyk et al., 2018; Fukasawa et al., 2011; Mäkipää et al., 2017). In our study, while representatives of Mortierellaceae were found in all studied environments, preferences toward certain microenvironments were observed on a lower taxonomic level. Entomortierella lignicola seems to be strongly associated with the mound environment, and representatives of Podila verticillata-humilis clade occur more often in the forest litter. The most plausible explanation of this variability is the difference in pH level between studied microenvironments. Linnemann (1941) stated (and later studies, such as Enghusen (1956) and Turner and Pugh (1961) confirmed) that the basic factor determining the occurrence of Mortierellaceae species is the soil pH. Coupling this characteristic with the observations of Jilková et al. (2012) that the pH of wood ant nest materials is typically higher than that of the surrounding forest soil, makes it a good explanation for the observed decreased abundance of P. verticillata-humilis strains in the ant-made environment. The pH in mounds can be too high for both Podila humilis and P. verticillata as Khalabuda (1973) isolated both species from forest soil and litter with pH 4.0-4.1 (P. verticillata) and pH 3.7-6.3 (P. humilis). Moreover, as species of P. verticillata-humilis clade are known to coexist with Pinus mycorrhiza (Khalabuda, 1973; Oh et al., 2019), their observed smaller abundance in the mounds could also be related to reduced amounts of alive plant roots in this microenvironment (Frouz & Jilková, 2008; Laakso & Setälä, 1998).

Entomortierella lignicola (previously Mortierella lignicola) is a species commonly isolated from decaying bark and wood, as well as soils, mostly forest ones (Giordano et al., 2009; Kuhlman, 1969; and based on metadata in GBIF and CBS culture collection). Therefore, the presence of this species in mounds is most probably related to the material of which the mounds are made. However, such a big difference in E. lignicola abundance between the litter and the mound suggests that ants actively stimulate the growth of this fungus or that the properties of the ant-made microhabitat particularly favor the development of this species. A hypothesis of adaptation towards the insect-made environments could be partially supported by previous isolation of E. lignicola from decayed wood with termite nests (Watanabe et al., 1998) and from the cadavers of F. polyctena ants (Siedlecki et al., 2021). Moreover, this species, belonging to the M. lignicola clade (sensu Wagner et al., 2013), is closely related to species also previously isolated from Camponotus and Formica ants and those ants’ infrabuccal pockets (Clark, 2002; Hyde, Norphanphoun, Abreu, Bazzicalupo, Chethana, et al., 2017; Siedlecki et al., 2021). Importantly, E. lignicola, E. beljakovae, and Mortierella formicae were also found in the infrabuccal pockets of F. polyctena (Siedlecki et al., 2022). Interestingly, all those ant-associated species are known to abundantly produce enlarged cells filled with oil droplets called gemmae (Wagner et al., 2013). We thus hypothesize that mycelium of this fungi could serve as a supplementary, nutrient-rich food source for ants, as also suggested for gemmae-producing Actinomortierella sp. aff ambiguus and fungivorous millipede (Macias et al., 2019). However, further studies focused on ants’ interactions with Entomortierella spp. are needed to verify those hypotheses.

Absidia fungi are found in forest soil, rhizosphere, and litter (Hesseltine & Ellis, 1964; Söderström, 1975), as well as in decomposing coniferous needles (Brandsberg, 1969), roots, and wood (Hesseltine & Ellis, 1964; Kuhlman, 1969). We thus expected representatives of this genus to be evenly distributed across all studied substrates. Therefore, the preference of Absidia cylindrospora clade representatives towards the interior of the mound is a surprising finding. Possibly higher temperatures in the mounds could allow for more abundant growth of these fungi as most species of Absidia cylindrospora clade are mesophilic, with the optimum growth
temperature between 25 and 34 °C (Hoffmann, 2010; Hoffmann et al., 2007). Moreover, Klamar et al. (2001) isolated some strains from this clade from compost piles, where environmental conditions are similar to the ones in mounds. Additionally, few species belonging to this clade, are known to be insect-related, with A. psychrophilia found in mycangia of ambrosia beetles (Hesseltine & Ellis, 1964) and A. cylindrosporatagether with A. spinosa isolated from the nests of Aphaenogaster texana carolinensis ants (Zettler et al., 2002). These results, when put together with our findings, suggest that representatives of this clade are adapted to survive in the proximity of ants.

Our results suggest that the mound community of Mucoromycota consists mostly of saprotrophic fungi commonly occurring in coniferous forest litter. However, the nest community is characterized by higher stability between seasons, increased abundance of Entomortierella lignicola and strains belonging to Absidia cylindrospora clade, and decreased abundance of Umbelopsis curvata and Podila verticillata/humilis clade. These findings suggest that F. polyctena ants’ mound could serve as a preferred microhabitat for the mound-associated species which could be otherwise repressed by other microorganisms present in the forest litter. However, which specific mound properties are the ones promoting the growth of these fungi mostly remain to be characterized, as well as the possible effect of the mound-associated Mucoromycota on the ant colonies.

References


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Figure legends

Figure 1. A mound of *Formica polyctena*.

Figure 2. Study scheme. The blue point on the map indicates the sampling area. The photo shows one of the *Formica polyctena* mounds sampled for the study. The circle on the photo of the mound indicates sampling...
from the depth of 15 cm, and the rings indicate sampling of the first 5 cm of the substrate. Blue arrows indicate the next step in the protocol. Abbreviations: MS - mound surface, MI - mound interior, FL - forest litter, SDA - Sabouraud Dextrose Agar, WA - Water Agar, Chl - chloramphenicol.

Figure 3. The averaged abundance of *Mucoromycota* regarding different substrates and seasons. The boxplots cover values between the first and third quartile, the middle line represents the median number of MM cfu per study variant, the whiskers represent maximum and minimum values below the upper and lower fence, and points represent outliers. The boxes marked with different letters are significantly different at p < 0.05 according to ANOVA with post hoc Tukey HSD tests.

Figure 4. The averaged *Mucoromycota* taxa diversity regarding substrate and sampling season. The boxplots cover values between the first and third quartile, the middle line represents the median number of MM taxonomic units per study variant, and the whiskers represent maximum and minimum values below the upper and lower fence.

Figure 5. Ordination of *Mucoromycota* communities obtained from studied samples, which is the result of two-dimensional ordination using NMDS, based on the Bray-Curtis dissimilarity matrix, computed from 24 taxa matrices transformed by Wisconsin double standardization. The colors of the points represent different substrates, and their shapes represent different seasons. Vectors represent species significantly shaping the composition of *Mucoromycota* communities (p < 0.05). Ellipses indicate 95% confidence intervals around centroids of different substrates.

Figure 6. Taxa occurrence in the study. A - Prevalence of taxa according to isolation substrate. Only taxa that were isolated from at least 50% of samples of any substrate are displayed on the graph. B - Number of cfu of taxa isolated in the study regarding isolation substrate. Taxa which were isolated less than 10 times are not displayed. In both graphs: taxa are arranged in descending order for the forest litter substrate, and ‘A. cylindrospora’ and ‘P. verticillata/humilis’ mean respectively representatives of *A. cylindrospora* clade and *P. verticillata-humilis* clade.

Appendix 1

Table A. Study sites metadata

Table B. List of representative strains

Table C. Raw Data

Data availability statement

Representative voucher specimens are stored in the Institute of Evolutionary Biology (University of Warsaw, Poland) collection (10% glycerol, -80°C) and the General Herbarium, University of Warsaw [WA] (dry specimens). Sequence data generated for this study are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). All reference numbers and taxon identification details are provided in Table B of Appendix 1. R script for statistical analysis is available online at https://github.com/mjkochanowski/mounds2024/blob/main/script. Raw data for the study are provided in Table C of Appendix 1.

Conflict of Interest Statement

The authors declare no conflicts of interest.

Author contributions

I.S. conceived the idea of the study. I.S., M.W., J.P and M.K. designed methodology; I.S. and M.K collected samples; I.S., M.K, M.W. and G.R. performed laboratory work, including molecular and morphological work; M.K. and I.S. performed statistical analysis; I.S. coordinated manuscript preparation and wrote the first draft of the manuscript; M.W., M.K. provided fragments of the manuscript; M.K. and I.S. prepared
figures; J.P. and A.O. edited the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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