

The bacterial and fungal communities in the nests of the ant *Formica exsecta*

Stafva Lindström

University of Helsinki
Faculty of Biological and Environmental Sciences
Organismal and Evolutionary Biology Research Programme

Academic dissertation

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Supervised by:

Professor Liselotte Sundström
Organismal and Evolutionary Biology
University of Helsinki, Finland

Dr. Sari Timonen
Department of Microbiology
University of Helsinki, Finland

Dr. Helena Johansson
Organismal and Evolutionary Biology
University of Helsinki, Finland

Reviewed by:

Dr. Jenni Nordén
Ecology and Evolutionary biology
Norwegian Institute for Nature Research (NINA)
Oslo, Norway

Dr. Sannakajsa Velmala
Natural Resources Institute Finland (Luke)
Helsinki, Finland

Examined by:

Professor Katarzyna Turnau
Institute of Environmental Sciences
Jagiellonian University, Kraków, Poland

Custos:

Professor Jouko Rikkinen
Organismal and Evolutionary Biology
Finnish Museum of Natural history
University of Helsinki, Finland

Thesis advisory committee:

Professor Anna-Liisa Laine
Organismal and Evolutionary Biology
University of Helsinki, Finland
Department of Evolutionary Biology and
Environmental Studies
University of Zurich, Switzerland

Dr. Minna Pirhonen
Department of Agricultural Sciences
University of Helsinki, Finland

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The more contact I have with humans, the more I learn.

T2, in Judgement Day

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Chapters I–III

This thesis is based on the following articles, which are referred to in the text by their Roman numerals.

- I. Lindström, S., Rowe, O., Timonen, S., Sundström, L. and Johansson, H. (2018): Trends in bacterial and fungal communities in ant nests observed with Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Next Generation Sequencing (NGS) techniques – validity and compatibility in ecological studies. PeerJ 6: e5289
- II. Lindström, S., Timonen, S., Sundström, L. and Johansson, H. (2019): Ants reign over a distinct microbiome in forest soil. In press, Soil Biology & Biochemistry.
- III. Lindström, S., Timonen, S., Johansson, H. and Sundström, L. (2019): Temporal variation of the microbiome in the nest mounds of the ant *Formica exsecta*. Manuscript.

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Stafva Lindström (SL), Helena Johansson (HJ), Owen Rowe (OR), Sari Timonen (ST), Liselotte Sundström (LS).

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Abstract

Biotic and abiotic characteristics shape the microbial communities in the soil environment. These characteristics vary both spatially and temporally, depending on variations in temperature, water availability and plant coverage, which on a larger scale are driven by topography, soil type, land use, vegetation and the ruling climate. In a sub-arctic climate, the seasonal fluctuations in temperature, precipitation and plant cover force the microbes in the top soil to adapt or decline.

For many organisms trying to survive the cold winters, a nest can provide protection from the harsh environment. For example, the mound building wood ants, such as the *Formica exsecta*, selectively choose both the spot, and the building material for their nest mounds, in which they overwinter. These ants are also able to generate extra heat by using their lipid reserves during early spring, to secure the brood development. The higher and steadier temperature inside the nest is further enhanced by the decomposition process, intensified by the constant addition of organic plant material carried out by the ants, and the availability of nutrients. This creates a unique microclimate inside the nest mounds, in which the microbes can stay largely protected from the environmental drivers of the microbial communities in the surrounding top soil.

I hypothesized, that the nest environment would promote the formation of unique bacterial and fungal communities in the nests, compared with the surrounding soil. I also hypothesized that the nest communities are stable in time, and that they would show less temporal than spatial variation. Furthermore, I expected to see nest-characteristic, temporally stable taxa, which could be identified as the core microbiome of the *F. exsecta* nests. However, to ensure a robust methodological approach, I first tested the combination of Next Generation Sequencing (NGS) and a classical community fingerprinting method, Terminal Restriction Fragment Length Polymorphism (T-RFLP).

I sampled nests of the mound-building ant *F. exsecta*, and generated NGS (Illumina MiSeq), and T-RFLP data of the bacterial and fungal communities. I used ordination techniques and network analysis to disclose the community structures in the nests, and compared them with the ones in the reference soil. I assessed the variation in diversity, evenness and enrichment of taxa in the nests, and evaluated their spatial and temporal variations. I also identified significantly nest characteristic and temporally stable taxa, which represent a potential core microbiome of the nests. The results show that the bacterial and fungal communities, in the rigorously curated nest environment, are significantly different from those in the reference soils. I demonstrate that the nests represent a niche, where microbial species can adapt and diverge from the communities in the surrounding soils. The findings in my thesis work contribute to our understanding of the composition, function and adaptation of microbial communities in patchy, secluded habitats, and open up for further studies in a multilayered ecological system.

Tiivistelmä

Vallitseva lämpötila ja kosteusolosuhteet vaikuttavat ratkaisevasti maaperän mikrobiyhteisöjen lajikoostumukseen ja toimintaan. Ilmasto-olosuhteet voivat kuitenkin vaihdella paljonkin, sekä ajallisesti että paikallisesti. Esimerkiksi Pohjolan ilmastossa vuodenaikaisvaihtelu on suurta, mikä edellyttää etenkin maaperän pintakerrosten mikrobeilta huomattavaa sopeutumiskykyä. Lisäksi maaperän mikrobit ovat jatkuvassa vuorovaikutuksessa muiden eliöiden kanssa.

Useat kylmässä ilmastossa elävät eliöt selviävät talvesta pesänsä turvin. Esimerkiksi loviniskamuurahainen (*Formica exsecta*) rakentaa neulasista ja muusta kasvinjätteestä monivuotisen pesäkeon, jonka alakerroksissa se selviytyy talven yli. Pohjoisilla leveysasteilla kylmä alkukesä aiheuttaa usein näille muurahaisille lisähaasteita: jotta seuraavan muurahaiskuvon kasvu ja kehitys onnistuisi lyhyen kesäkauden aikana, on pesän sisäinen lämpötila pidettävä tasaisena alkukevään hallaöistä huolimatta. Pesärakennelman hitaan lahoamisprosessin tuottama lämpö ei tähän yksin riitä, vaan lisäksi vaaditaan pesän työläisiltä kykyä muuntaa rasvavarastojaan lämmöksi. Ulkolämpötilaa hieman korkeampi ja tasaisempi lämpötila pitää pesän sisuksen kuivana, vaikka pesän ulkopuolella kosteusolot vaihtelevat. Kesäaikaan muurahaiset kunnostavat pesäänsä jatkuvasti kantaen kekoon uutta rakennusmateriaalia, joka pitää lahoamisprosessin käynnissä, mutta ne myös kuljettavat jätteitä ja taudinaiheuttajia pois pesästään. Pesärakennelman sisällä suotuisat olosuhteet edesauttavat mikrobiyhteisöjen talvehtimistä ja voivat mahdollistaa myös sellaisten mikrobien runsastumisen, joiden esiintymistä pesän ulkopuolinen lämpötilan vaihtelu rajoittaisi. Pesän mikrobit ovat jatkuvassa tiiviissä vuorovaikutuksessa muurahaisyhteisön kanssa, mikä muokkaa mikrobiyhteisöjä ja luo edellytyksiä niiden erikoistumiseen.

Väitöskirjani tarkoituksena on selvittää, millä tavoin loviniskamuurahaisen pesärakennelman sisällä olevat mikrobit eroavat pesän ulkopuolella olevista mikrobiyhteisöistä, sekä arvioida pesän sisäisten yhteisöjen ajallista ja paikallista pysyvyyttä. Yhdistämällä kaksi molekulaarista menetelmää tutkin loviniskamuurahaisen pesärakennelmissä olevien bakteeri- ja mikrosieniyhteisöjen rakennetta, lajidiversiteettiä ja verkostoja sekä niiden eroja pesän ulkopuolella oleviin yhteisöihin. Lisäksi selvitin pesärakennelmalle tyypillisen mikrobiomin koostumuksen.

Tämän väitöskirjan keskeisin tulos on havaittu merkittävä ero loviniskamuurahaisen pesien sisäisten ja niiden ulkopuolisten mikrobiyhteisöjen välillä. Tulosten perusteella muurahaisten huolellisesti vaaliman pesärakennelman sisällä mikrobiyhteisöt muokkautuvat sekä lajikokoonpanon että lajien runsauden suhteen eri suuntaan kuin sen ulkopuolella olevat yhteisöt. Pesärakennelman sisällä mm. useat muurahaisille symbioottiset bakteerisuvut yleistyvät. Kokonaisuudessaan tutkimukseni edistää lajien välisten vuorovaikutusten sekä saarekemaisissa elinympäristöissä elävien mikrobiyhteisöjen sopeutumis- ja erikoistumiskyvyn parempaa ymmärtämistä.

Summary

Stafva Lindström
Evolution, Sociality and Behaviour
Organismal and Evolutionary Biology Research Programme
Faculty of Biological and Environmental Sciences
University of Helsinki

1 Introduction

1.1 Microbes in soil

Microbes are ubiquitous in soil and often occur in extremely diverse communities, with complex networks of interaction and functional traits. The distribution of microorganisms in soil is heterogeneous, and largely controlled by the biotic and abiotic characteristics of soil (Franklin and Mills, 2003; Garrido-Jurado et al., 2011; Quesada-Moraga et al., 2007). These characteristics vary both spatially and temporally, depending on climatic factors, topography, soil type, vegetation systems and land use (Ettema, 2002), which cause variation in temperature (Kennedy et al., 2006), water availability (Hawkes et al., 2011; Rousk and Bååth, 2007), land management (Edel-Hermann et al., 2004), plant coverage (Ladygina and Hedlund, 2010; Oddsdottir et al., 2010; Prescott and Grayston, 2013) and plant species (Leff et al., 2018). The spatial distribution of microbes encompasses horizontal clustering, mainly occurring in the upper soil layers, and vertical clustering, which is due to the stratification (layered structure) of soil (Kadowaki et al., 2014). Temporal variations are predominantly driven by seasonal changes, either directly through seasonal variation in temperature and rainfall, or indirectly, for example via seasonal changes in plant coverage and community composition, and photosynthetic activity (Baldrian, 2017a; Kivlin and Hawkes, 2016).

1.2 Drivers of the structure and diversity of microbial communities in soil

The level of moisture in soil shapes the community composition and function of bacteria and fungi (Evans and Wallenstein, 2014; Hawkes et al., 2011; Jarvis et al., 2013; Sorensen et al., 2013). The variations in the soil water content affect soil acidity, which both directly, and through nutrient availability strongly influence the microbial communities (Kaiser et al., 2016; Nacke et al., 2016; Tecon and Or, 2017). Both the availability of C, the mineralization of P, and the N cycle are determined by seasonal variations (Lauber et al., 2008; Rasche et al., 2011; Strickland et al., 2009; Zhou et al., 2017). Manipulation of soil, whether it is tillage or compaction carried out by humans (Hartmann et al., 2014), or bioturbation performed by animals, such as ants during nest construction (Nkem et al., 2000), affects the composition and dynamics of the microbial community.

The abundance and function of soil microbes is also largely determined by plants, in particular, trees (Kaiser et al., 2016; Prescott and Grayston, 2013; Zhou et al., 2017). The carbon rich plant root exudates attract several bacteria and fungi (Lladó et al., 2017), whereas the large amount of organic matter produced by trees and understory plants provides resources for the decomposers (Baldrian, 2017b). In boreal forest soil, the communities of mycorrhizal fungi are also highly governed by ericoid plants (Sietiö et al., 2018; Timonen et al., 2017). In areas with pronounced seasonal variations

in the vegetation, the fungal decomposers are abundant in the early spring and later in the autumn, whereas mycorrhiza forming fungi are more dominant during the photosynthetically active summer period (Santalahti et al., 2016; Žifčáková et al., 2016).

The temperature has a pronounced effect on microbial community composition (Zhou et al., 2016). The temperature affects the survival of the microbial cells both directly and indirectly through the availability of liquid (non-frozen) water. Many of the microbial processes slow down or cease in temperatures close to zero, and only taxa with high tolerance of cold can survive, or avoid severe decline in sub-zero temperatures (Margesin and Miteva, 2011; Rankinen et al., 2004). In particular, soil bacteria and fungi in a boreal forest have to adapt, to survive in the harsh climate (Männistö et al., 2018), where summers are short and the periods of sub-zero temperatures long. Many organisms, both vertebrate and invertebrate, are only able to survive and reproduce in the cold climate due to their niche-construction or nest-building abilities (Barber, 2013; Kylafis and Loreau, 2011).

1.3 The nest

Building of nests to shelter offspring is of critical importance, for vertebrates and invertebrates alike. Therefore the concept of nest building, including both behaviour and nest design, is expected to be under strong natural selection (Barber, 2013). Nests create “islands” in the ecosystem, providing different prerequisites for the species composition of the microbial communities, compared to those in the surrounding environment (Jouquet et al., 2006). The material and architecture of a nest or a dwelling creates a physicochemical framework, in which the host shapes the nest-characteristic microbiota, but where the nest microbiota in return influences the fitness of the hosts, may those be ants, birds or bees (Brandl et al., 2014; Broussard and Devkota, 2016; Voulgari-Kokota et al., 2019). Much like the microbiota of the human gut (Coyte et al., 2015), or the plant root microbiome (Beckers et al., 2017), the microbiome of a nest is often distinct from the one in the surrounding environment, with respect to both community composition and species abundance. For example, the nest material of birds, like that of the reed warbler, often facilitates bacteria originating from environmental sources, such as plants, soil, food remains, or material from the host itself, such as the plumage (Brandl et al., 2014). The incubation period before the chicks hatch, however, significantly changes the bacterial assemblage in the bird nest, reducing several potentially harmful bacterial groups, possibly due to the sanitation activities of the parents, or because of the antibiotic properties of the eggs (Brandl et al., 2014). Some termites build huge nest constructions, which reach several meters above and below the ground. When building the nest walls, they aggregate the soil by mixing it with their faeces. These worker-made micro-aggregates form a distinct microbial environment, the termitosphere, which maintains beneficial lignocellulolytic and antibiotic microorganisms in the nest environment (Moreira et al., 2018).

1.4 The wood ant nest

The nests of wood ants may not be as conspicuous as termite domes, but depending on the species, wood ant nests range from large mounds above ground to complex excavated underground dwellings. The nests of mound-building wood ants provide a well-functioning shelter from environmental fluctuations, while demonstrating the ecosystem engineering skills (*sensu* Jouquet et al., 2006) of ants. The nest site is not chosen randomly, instead many wood ants carefully select sunlit, open spots for their nest, in order to secure enough insolation during the summer (Katznerke et al., 2010;

Littlewood and Young, 2008). The nest material, mainly litter consisting of coniferous needles, and pieces of moss and lichen collected from the surrounding forest floor, is also chosen selectively (Kilpeläinen et al., 2007). This selectivity when choosing the building blocks for the nest often also includes pieces of coniferous resin, known to have antimicrobial qualities (Brütsch and Chapuisat, 2014; Castella et al., 2008; Christe et al., 2003). Furthermore, ants restrict the amount of harmful microbes by cleaning the nest, grooming each other (Otti et al., 2014), and by producing antimicrobial compounds themselves (Stow and Beattie, 2008). Many ant species also establish symbiotic associations with beneficial microbes to fight pathogens (Vander Meer, 2012), whereas some ants take the co-operation a step further, by actively farming fungi for food (Kellner et al., 2015; Mueller et al., 2001). Unlike the fungus-farming ants, or the predatory ant species, the mound building wood ants transport considerable amounts of aphid-derived honeydew into the nest for food. The accumulated food residues in the nest reflect the species-specific feeding habits of the host, which contributes to why the microbial nest communities are assumed to reflect the behaviour of the hosting ant species (Boots et al. 2012). For example, the nests of predatory ants are enriched in prey-originating nitrogen, whereas honeydew, the major nutrition source of many *Formica* ants (Domisch et al., 2009), creates a surplus of sugar in the nest. This intensifies respiration and increases decomposition, and the following release of basic cations affects the microbial community through changes in the pH (Jilková et al., 2012; Jilková and Frouz, 2014; Kaiser et al., 2016).

For some *Formica* species living in the northern hemisphere, where summers are short and temperatures fluctuate, the nest represents a thermoregulated environment, necessary for prolonging the breeding and foraging period (Jurgensen et al., 2008; Rosengren et al., 1987). During the winter, when the temperature in the upper soil layer drops below zero (Rankinen et al., 2004), the ants stay inactive in the inner parts of the nest. In early spring, the workers create heat by utilizing their lipid reserves, thereby increasing the temperature in the nests. In early summer, the temperature can still drop well below their comfort zone, which could endanger brood development without additional generation of heat (Rosengren et al., 1987). The high and steady temperature, further maintained by the decomposition process of the accumulated organic material in the nest mound (Laakso and Setälä, 1998), keeps the inside of the nest drier than the outside environment (Domisch et al., 2008; Jilková and Frouz, 2014), thereby influencing the microbial composition (Duff et al., 2016).

Ant activities, such as the ones listed above, create a unique microclimatic environment. The nest environment of mound-building ants is hence liable to influence the species composition and abundance of the bacterial and fungal communities, and diverge their adaptive trajectories from the one in the background environment. Particularly in the sub-arctic climate of a boreal forest, the nest could allow the microbial communities to stay largely unaffected by the typical seasonal shifts in the temperature, precipitation and plant cover, which affect the surrounding top soils. In previous studies, the microbial biomass has been found to be significantly higher in the nests of many ant species, compared to the outside reference soil (Boots et al., 2012; Dauber et al., 2001). Furthermore, a higher number and diversity of fungal taxa was detected inside the nests of *Formica* ants, compared to the non-nest soil (Duff et al., 2016). None of these studies were, however, performed in a boreal climate zone.

1.5 Microbial association with the nest material and the nest host

The type of material used by ants to build their mounds explains, to a certain extent, the presence or absence of many microbial taxa in the nest. *Formica* ants frequently use pine and spruce needles, and pieces of moss and lichen as nest material (Laakso and Setälä, 1998; Lenoir et al., 2003; Littlewood and Young, 2008). Concentration of such material would be expected to enrich certain microbial taxa in the nest, for example guilds of specialized decomposers of recalcitrant litter (Žifčáková et al., 2017), or endophytes of coniferous needles (Saikkonen, 2007). The dryness and continuous perturbation and rebuilding keeps the nests mainly void of live plants, which in turn prevents the occurrence of mycorrhizal fungi and thereby also the presence of any associated mycorrhiza helper-bacteria (Lladó et al., 2017). The absence of plants also restricts plant-associated bacterial nitrogen fixers from settling in the nest mounds (Boots et al., 2012), whereas many ant species form symbioses with nitrogen fixing bacteria themselves (Russell et al., 2009).

Many – if not all – ant species establish symbiotic relationships with microbes, both in their guts and on their cuticle (Anderson et al., 2012; Hu et al., 2014; Marsh et al., 2014; Mattoso et al., 2012; Russell et al., 2009). Bacteria, mainly members of the Actinobacteria, are involved in several symbioses, in which the ant host or its food is protected by the beneficial symbiont against pathogenic infections (Reyes and Cafaro, 2015). For example, in their study of 29 insect species (including 10 ant species) Matarrita-Carranza et al. (2017) showed that the associations between Hymenopteran species and Actinobacteria are much more prevalent than previously described. It has also been suggested that associations with beneficial bacteria are sometimes linked with the more vulnerable stages of the insect life cycle, the larvae and pupae (Seipke et al., 2012). It was thought that these symbiotic bacteria were transmitted only vertically (from parent to offspring), but due to the social behaviour of ants, with their dense colonies, shared food and communication between individuals being based on physical contact, it is now thought that horizontal transmission (among individuals of the same generation) is common as well (Kaltenpoth and Engl, 2014). In some cases, the transmission of bacteria from adult workers to newly eclosed ones is limited to a very narrow time window for obtaining a successful transfection. Some ant species also seem to selectively pick certain bacterial strains, in this case Actinobacteria, from their immediate surroundings (Reyes and Cafaro, 2015). This suggests that the formation of a successful symbiotic association requires the presence of the symbiotic microbe either within, or close to, the nest (Kaltenpoth and Engl, 2014).

1.6 Developing the combination of T-RFLP and NGS (Illumina MiSeq)

Longitudinal studies on microbial communities require the development of reliable and cost effective methodologies. Several techniques have successfully been used for fingerprinting complex microbial communities, but the semi-quantitative properties (Blackwood et al., 2003), high reproducibility, low cost (Thies, 2007) and the technical and analytical straightforwardness of T-RFLP (Terminal Restriction Fragment Length Polymorphism) has cemented it as one of the leading fingerprinting methods for several decades (Schütte et al., 2008). However, in order to identify the microbes taxonomically, T-RFLP has usually been combined with preparation and sequencing of clone libraries (van Elsas and Boersma, 2011), which is both time-consuming and expensive. The option of using Next Generation Sequencing (NGS) to gain taxonomic and abundance information of a large longitudinal dataset is generally too costly. Complementing T-RFLP with NGS techniques can offer a convenient way of generating and analyzing metagenomic microbial data, including the addition of

taxonomic information achieved by NGS sequencing, to the T-RFLP fingerprints. The few studies to date on bacteria, in rumen (De La Fuente et al., 2014), in Antarctic soil (van Dorst et al., 2014), in tropical soil (Supramaniam et al., 2016), all indicate that the patterns and trends in data generated by the two techniques are largely congruent. Studies that utilize both T-RFLP and NGS data to explore fungal communities are scarce in the literature, as are studies comparing both bacterial and fungal communities using both techniques. The protocols and methods used for microbial NGS analysis are still also far from standardized and NGS datasets often require complex bioinformatic interpretation (Balint et al., 2016; Tedersoo et al., 2015).

2 Aims of thesis

The overall aim of my thesis was to assess the bacterial and fungal communities in the nests of the ant *Formica exsecta* (Nylander 1846). I predicted that ant activities, such as their building and nesting habits, together with the biotic and abiotic characteristics of the nest mound, would influence the bacterial and fungal communities in the nests. Many microbial taxa may be highly adapted to the nest environment, which should manifest itself as a difference in the composition, frequency, or abundance of these taxa, compared to the ones in the background soil. Furthermore, the shielded nest environment could allow the microbial communities to stay largely unaffected by the typical seasonal drivers that shape the structure of microbial communities in boreal forest soil. This may increase the temporal stability of the communities and set them off to trajectories of adaptation, which diverge from those ruling in the background soils.

In Chapter I, I test the hypothesis that T-RFLP and NGS are both well suited separately for the examination of complex microbial communities in soil, but that they make an even more robust and informative data set when combined. For this, I sampled the microbial communities inside the nest mound of *F. exsecta* over a two-year period and generated data of spatiotemporal patterns of abundance and species composition, diversities and sampling efficiencies by using both methods. I then combined this pilot survey of the microbial communities with an evaluation of the overall suitability of the methodology for the study, and assessed the congruence of the results obtained with both methods.

In Chapter II, to test the hypothesis that the constantly curated and maintained environment in the *F. exsecta* nests promotes diverging bacterial and fungal communities from those in the surrounding top soil, I examined the communities in these two environments, regarding their species composition, abundance and patterns of correlation. In this chapter, I also identified a set of nest-characteristic bacterial and fungal taxa, which are likely to represent the core microbiome of the nests, and I evaluated the function of these taxa, as well as their potential associations with the nest host.

My hypothesis for Chapter III was that the shielded nest environment alleviates some of the temporal effects, which drive the microbial communities in the surrounding boreal top soil. This would allow for less temporal than spatial variation in the *F. exsecta* nests, manifested as consistent taxa and a low level of species turnover. To test this, I assessed the level of within-season and between-season resilience of bacterial and fungal taxa in the nests, and considered the potential functions of the most consistent taxa in the nest environment, over a three-year sampling period. I also examined if I could detect these taxa equally frequently and abundantly, during one season, in the surrounding soils.

Finally, I compared the bacterial and fungal species turnover dynamics, which were detected during one season.

3 Material and methods

3.1 Study site, the study objects and sampling

The study site, the two islands of Furuskär and Joskär, is located in the SW archipelago of Finland, in the proximity of the Tvärminne Zoological station (59°84'196"N, 23° 20'182"E), where the *F. exsecta* populations have been monitored since 1994 (Sundström et al., 1996; Vitikainen et al., 2011). The biotopes are typical of the SW coast of Finland, encompassing a mix of granite outcrops, pine spinneys, dry meadows, and lush patches of vegetation. The soil classifies as leptosol (<http://www.fao.org/soils-portal/soil-survey/soil-classification/world-reference-base/en/soil>) of varying thickness, mostly between 20 and 50 cm, depending on topographic factors. The thickness of the upper litter layer varies as well, mainly between 5 and 15 cm, being thicker in the immediate proximity of coniferous trees. In general, the vegetation is typical of a boreal forest, dominated by Scots pine and Norway spruce, together with ericaceous shrubs and *Deschampsia* and *Festuca* grasses, but the species composition of the plants in the close proximity of each nest varies, both spatially and temporally. The nests are scattered over the two islands, mostly situated at least some 50 m from each other. Overall, the main characteristic shared by all the sampled nest locations was that the nests were built in open and dry spots.

In such forest soils, fungi generally dominate over bacteria in the microbial biomass, due to their ability of hyphal growth by which they can bridge air-filled spaces between the soil particles, enabling the transport of water and nutrients over considerable distances. The fungal dominance in forest soils is further enhanced by monopolizing the formation of mycorrhiza and the decomposition of lignocellulose (De Boer et al., 2005). The decomposition of dead plant biomass is primarily mediated by fungi, whereas bacteria are responsible for decomposition of the fungal mycelia (Lladó et al., 2017). Soil fungi and bacteria are likely to come into competition over resources (Rousk and Bååth, 2007; Rousk and Bååth, 2011), yet they may also form mutually beneficial associations (Lladó et al., 2017). Many, if not most, of the complex relationships between soil bacteria and fungi are still unknown, as is their contribution to the formation and function of the microbial community (Frey-Klett et al., 2011).

Ants of the genus *Formica* are common in Northern Eurasia (Czechowski, Radchenko and Czechowska, 2002, Collingwood, 1979). It is a territorial and aggressive genus, feeding on a variety of food sources, such as other invertebrates and honey dew derived from tree aphids (Erős et al., 2009). The ant *F. exsecta* inhabits meadows and dry woodlands, and prefers sunny openings (Czechowski et al., 2002), where they construct their above-ground nest mounds. The nest mounds are usually rather small, often less than 30 cm in diameter, but nests larger than one meter in diameter have also been recorded (Czechowski et al., 2002). The nests are built of plant litter gathered from the surroundings, sometimes with small additions of mineral soil (Jurgensen et al., 2008). The average lifespan of the perennial nests has been estimated at 6.5 years (Haag-Liautard et al., 2009), but a nest can stay active up to 30 years (Pamilo, 1991). The active nests in our study area were void of live

plants, but some vegetation is known to occur in nests that are located in grasslands (Schütz et al., 2008).

I sampled nests of *F. exsecta* over the period of three years in 2013–2015, and in 2015, also corresponding reference samples from the soils surrounding the nests, monthly, during May to September. DNA extracted of all samples was subjected to T-RFLP, and a subset of samples was furthermore sequenced by Illumina MiSeq. The T-RFLP analysis, and the Illumina MiSeq sequencing, the bioinformatics and the taxonomic classification procedures are explained in detail in Chapter I.

3.2 T-RFLP and NGS combined, testing of sampling success, relative abundance patterns and virtual T-RFs

The microbial communities in soil are notoriously complex, due to their vast diversity and the large number of unidentified species they encompass (Zhou et al., 2015). Analysing them in any substrate is demanding, and as there were no previous studies available on the communities in *F. exsecta* nests, it made the choosing of proper methods particularly challenging. As there were very few well-documented protocols available for investigating soil metagenomics, the suitability of the combination of methods had to be tested for the study at hand. To verify the compatibility of the methods, and how well combining them was suited for examining the target communities I compared the results gained from both T-RFLP and Illumina MiSeq, of functional, abundance and diversity patterns of the bacterial and fungal communities (Chapter I). To ensure that the sampling and sequencing capture a sufficient proportion of the population, I performed a Good's estimate analysis (Good, 1953), which I visualized by species accumulation curves, of data acquired by both methods. I also evaluated the similarity of the variations caused by spatial and temporal effects, obtained by both methods. Furthermore, I investigated the extent to which T-RFLP fingerprinting can be complemented with taxonomic information obtained from NGS data. I compared the relative abundances of T-RFLP and NGS data by correlating the total number of Terminal restriction fragments (T-RFs) with an equivalent number of OTUs to ensure that they showed similar proportions of abundance (Chapter I). I performed a virtual cutting of the OTU sequences at the restriction sites of the enzymes MspI and HaeIII, and, based on the length of the fragments, I matched them with the T-RFs of the same length (number of base pairs). The species identities of virtual T-RFs derived from the OTU data were then transposed to the experimental T-RFs, as explained in detail in Chapter I.

3.3 The dissimilarity and diversity patterns, networks and species turnover dynamics

To examine the level of similarity between the microbial nest communities, between the nest communities and communities of the reference soils, and between the different sampling times, I created Bray-Curtis dissimilarity matrices for both the T-RFLP and NGS data. I visualized the similarity distances by plotting them into Principal Co-ordinates Analysis (PCoA), and I tested the dissimilarities for the effects of 'nest', 'month' (Chapters I, II and III), 'island' (Chapters I and II), 'year' (Chapters I and III) and finally 'environment' (nest or reference soil, Chapter II). I also explored the correlation patterns of the bacterial and fungal communities across the nests and the corresponding reference soils by correlation networks (Faust et al., 2015b), and I tested the effect of environment (nest or reference soils,) on the total read abundance in Chapter II. To determine the levels of diversity of the microbial communities, I calculated diversity indices (Shannon-Wiener's

H') and counted the numbers of OTUs, T-RFs (Chapters I, II and III) and the number of identified taxa, (Chapter III), calculated estimates of evenness (Chapters II and III), and examined the variation in these parameters caused by spatiotemporal effects. Furthermore, to evaluate the structure of the communities, I estimated their level of functional organization (Marzorati et al., 2008; Wittebolle et al., 2009), (Chapters I and II), and I measured the appearance and disappearance of species (species turnover rates) per year and month (Hallett et al., 2016), in Chapter III.

3.4 Indicator taxa and consistent taxa, and their potential associations with the nest material or the nest host

In order to identify the bacterial and fungal taxa that were significantly indicative of the nests compared to the reference soils, I performed an indicator analysis (Dufrêne and Legendre, 1997) (Chapter II). Furthermore, to determine the taxa that were consistently present in the nests, I identified the species which were detected in all samples, at every sampling occasion following Shade and Handelsman, 2012). In Chapters I, II and III, I discuss potential functional patterns, based on the taxon-specific traits associated with the microbes in question.

4 Results and discussion

4.1 Developing and testing the methodology

The study of soil bacteria and fungi is challenging, because the communities are extremely complex, many of the techniques are not yet standardized, and several methods are still untested for bias or remain poorly documented (Amend et al., 2010; Bálint et al., 2014; Knight et al., 2018). Different environments facilitate microbes of different composition and diversity, and the methodology should hence be tested for, and adapted to, the specific microbial target community at issue (Buttigieg and Ramette, 2014; Faust et al., 2015a; Weiss et al., 2016). In the study of microbial communities, acquiring high-quality data on a sufficient amount of samples is crucial for obtaining unbiased results. There may also be considerable differences between kingdoms (bacteria and fungi) in sampling and sequencing success. In our study, the percentage of fungal diversity captured by T-RFLP was slightly lower than the bacterial. The fungal primer pair targeted a shorter DNA-fragment than the bacterial primers did, which provided less enzymatic cutting sites, thereby allowing for less variation. Alternatively, the fungal communities in Chapter I were slightly undersampled, compared to the bacterial ones. The consistently observed lower levels of fungal diversity (Chapters I, II and III), based on T-RFLP and NGS data alike, could indicate that the fungal communities in fact were much less diverse than the bacterial ones (De Vries and Shade, 2013; Wagg et al., 2014).

In Chapter I, I show that the sampling success of Illumina MiSeq was very good and in good correspondence with recent NGS studies of soil microbes (Lazzaro et al., 2015; Meiser et al., 2014), whereas the sampling by T-RFLP was moderate, but still in the same magnitude as other comparable studies (Blackwood *et al.*, 2003; Schwarzenbach, Enkerli and Widmer, 2007; Frey *et al.*, 2009; Anderson *et al.*, 2011; Boots *et al.*, 2012; van Dorst *et al.*, 2014, Fig 1).

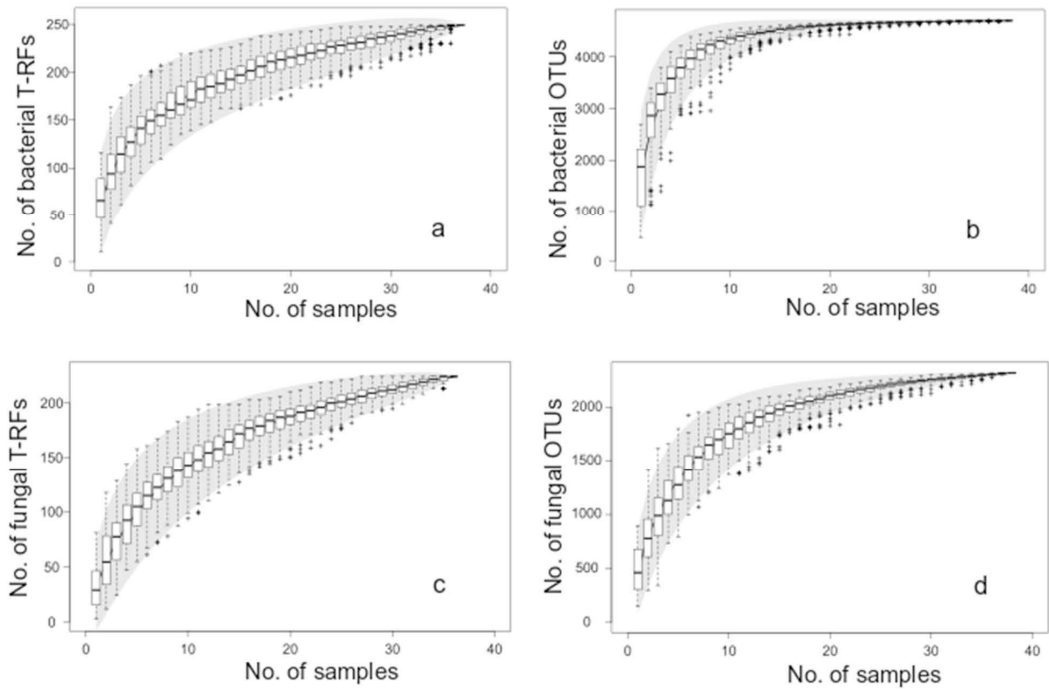


Figure 1. Species accumulation curves for bacterial T-RFs (a) and OTUs (b), and fungal T-RFs (c) and OTUs (d). The line represents the actual sampling, the grey area depicts the standard deviation, and the box plot shows the species richness based on linear intrapolation of random permutations. Copyright©2018 by PeerJ (reproduced from Chapter I, Lindström et al., 2018. PeerJ 6, e5289).

Table 1. PERMANOVA tests of the temporal (year and month) and spatial (island and nest) effects on the Bray-Curtis dissimilarities of the bacterial and fungal T-RF and OTU data (2013–2014). Copyright©2018 by PeerJ (reproduced from Chapter I, Lindström et al., 2018. PeerJ 6, e5289).

Test effect	T-RFs				OTUs			
	<i>F</i>	<i>df</i>	<i>R</i> ²	<i>p</i>	<i>F</i>	<i>df</i>	<i>R</i> ²	<i>p</i>
	Bacteria							
year	1.08	36	0.030	0.336	0.49	37	0.014	0.873
island	2.50	36	0.067	0.021	6.84	37	0.160	0.001
nest	3.58	36	0.093	0.001	4.20	37	0.104	0.005
month	2.55	36	0.068	0.020	1.32	37	0.035	0.210
	Fungi							
year	1.12	35	0.032	0.284	0.85	37	0.023	0.555
island	2.99	35	0.081	0.001	7.46	37	0.172	0.001
nest	4.65	35	0.120	0.001	7.31	37	0.169	0.001
month	0.94	35	0.027	0.558	1.01	37	0.027	0.378

The correlation between the Bray-Curtis distances according to the T-RFLP data, and the ones of the NGS data revealed a slightly lower correspondence between the two matrices, compared to the ones in e.g. De La Fuente et al. (2014) or Piloni et al. (2012). Both techniques captured similar patterns of variation (Chapter I), showing that the communities were primarily structured at the nest level, although some structure was found at the level of island as well (Table 1). The overall taxonomic information obtained by the virtual T-RFLP was largely comparable to those reported for studies involving clone libraries (Axelrood et al., 2002; Lin et al., 2011; Youssef and Elshahed, 2009).

The prices of NGS analyses have lately become more amenable, which nowadays have made these methods highly popular, resulting in huge amounts of published studies. However, as the costs for the actual sequencing have gone down, the demand for bioinformatics expertise has increased dramatically. This new “technological ecology” requires high computational and statistical skills from biologists. The challenges today lie in identifying the caveats of the NGS-based methods and choosing proper downstream analysis. In particular, the possible biases in connection with library construction and assembly are not always understood (Knight et al., 2018). For example, using the number of sequenced reads as a measure of abundance is not straightforward, although it has become common practise in a majority of NGS-based studies. Furthermore, the normalization or rarefying (downsizing) of the data always entails loss of statistical power (Hugerth and Andersson, 2017), and discriminates against the rare species.

During the whole study, I derived NGS data from seven nests, four on Furuskär and three on Joskär. In addition, I produced T-RFLP fingerprints of 15 nests on Furuskär, and 19 nests on Joskär, to inform the NGS data (Table 2). Taken together, both methods are suitable for the analysis of microbial communities in challenging media, but when combined, they complement each other well (results in Chapters I, II, III). T-RFLP can be trusted to detect the same community patterns as Illumina MiSeq, and it requires a lot less bioinformatics. A combination of the two molecular methods is a good alternative, when a robust and cost efficient method is required, in particular for comprehensive longitudinal studies.

Table 2. The nests from which NGS data were derived, and the number of nests from which T-RFLP data were derived.

		Chapter I	Chapter II	Chapter III
Nests, from which NGS data were derived:				
Island: Furuskär	F12	X	X	X
	F42		X	
	F103		X	
	F120	X		
Island: Joskär	J30	X	X	X
	J40	X	X	X
	J93		X	
Number of nests, from which T-RFLP data were derived:				
Island: Furuskär		4	8	3
Island: Joskär		4	12	3

4.2 The distinct nest communities

In Chapter II, I show that both the bacterial and fungal nest communities are highly distinct from the reference soils, throughout the sampling season. Both communities were mainly structured according to the environment in question (nest or reference soil), suggesting that the nest environment promotes the formation of unique communities (Fig 2). The community patterns also revealed secondary effects of dissimilarity clustering; those explained by the physical location of each nest and its corresponding reference soil pair, indicating that the immediate surroundings, probably through the vegetation, also influence the communities. The sequenced OTU-data, and the larger T-RFLP data set both showed comparable results. The networks also displayed clear clustering according to environment and location, rendering further indication of an environment-dependent community assembly, for both bacteria and fungi (Fig 3). Both the nest communities and the communities in the reference soils suggested similar patterns of functional organization, signalling dominance of a few species. This suggests that the communities may encounter difficulties when attempting to regain their original function after disturbance, due to the low evenness of species (Marzorati et al., 2008). However, the analysis of functional organization should be interpreted as an indicative characterization of the traits and function of the microbial community. These communities often encompass microbes belonging to different taxonomic groups, but with similar functions (Burke et al., 2011; Shade and Handelsman, 2012). A study specifically targeting the function of the most characteristic microbes in the nest and the reference environment would be needed to clarify this.

Most of the bacterial and fungal phyla were present in both environments, but the abundances of several phyla differed between the nest and the reference soils. In particular, the enrichment of taxa belonging to Actinobacteria and Ascomycota in the nests could well be supported by the coexistence of ants, whereas the phylum Glomeromycota was detected only in the reference soils, likely because this phylum contains several mycorrhiza forming taxa (Young, 2012), which are dependent on live plant roots.

I identified a set of indicator taxa, which were significantly more frequent and abundant in the nests compared to the reference soils. These taxa represented largely the same bacterial and fungal genera, which were also significantly enriched in their respective environment (Chapter II). Those taxa, which achieved an indicator status for all three sampled months, were considered to represent the core indicators, or the microbiome (sensu Shade and Handelsman, 2012) of the nests. The core indicators of the nest encompassed several taxa, which in previous studies have been found to be significantly associated with ants, such as the bacterial genera *Pseudonocardia* (Folgarait et al., 2011), *Streptomyces* (Sen et al., 2009) *Burkholderia*, *Methylobacterium* (Van Borm et al., 2002), and *Brevundimonas* (Jaffe et al., 2001), and the fungus *Exophiala* (Duarte et al., 2014). These taxa may well represent members of the potential *F. exsecta* microbiome, possibly with the ability to form symbiotic relationships with the ants. In addition, the core indicators of the nest included taxa that may owe their presence there to the typical material that the *Formica* ants pick for nest construction. The plants, mosses and lichens from which the nest material originates (Laakso and Setälä, 1998), are often associated with the bacterial genera *Frondehabitans* (Han et al., 2016), *Methylobacterium* (Pirttila et al., 2000), and Cyanobacteria/Chloroplast (Aschenbrenner et al., 2016), and the three fungal genera, *Oidiodendron*, *Scleroconidioma* (Davey and Currah, 2006), and *Umbelopsis*.

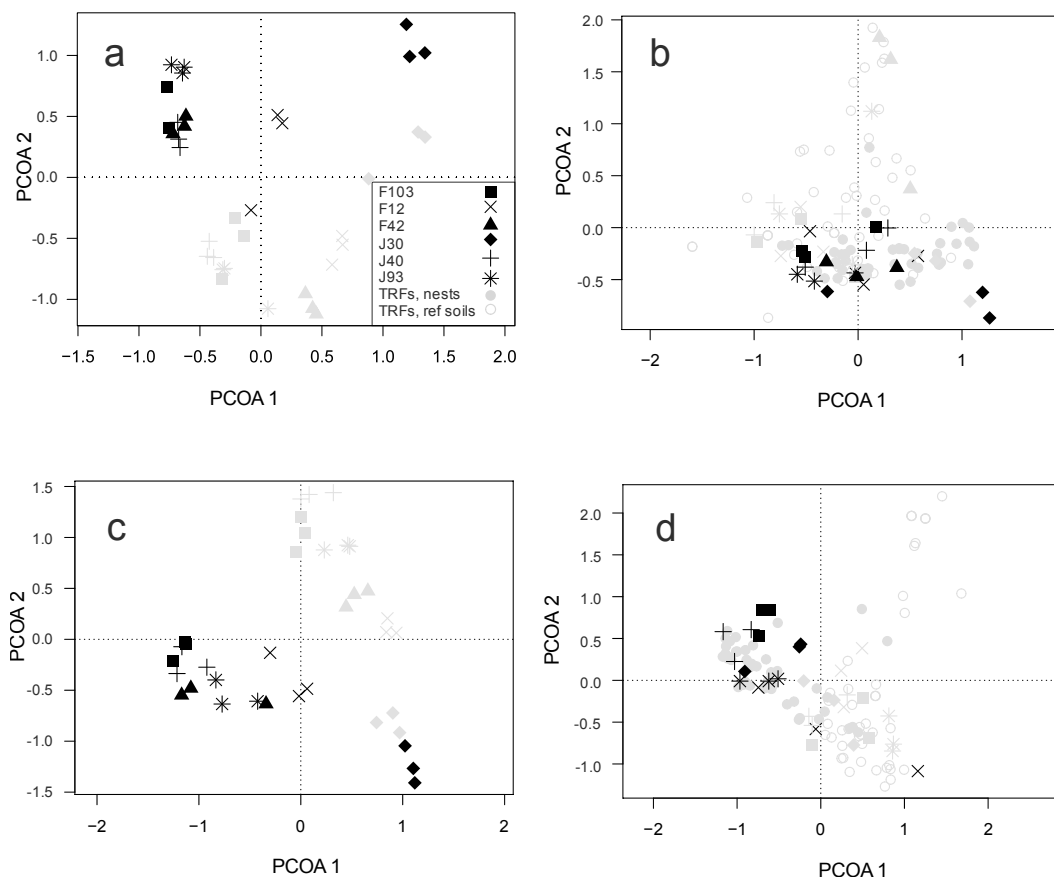


Figure 2. Distribution of nests and reference soil communities according to Principal Coordinates analysis, (a) bacterial OTUs (a), n=36, (b) bacterial T-RFs, n=127, (c) fungal OTUs, n=36, and (d) fungal T-RFs, n=121. Different symbols represent the location of each nest sample and corresponding reference soil sample. For the OTU data and the corresponding samples in the T-RF data, black symbols indicate the nest environment, and corresponding grey symbols indicate the reference soil environment. The closed grey circles represent the remaining T-RF samples of the nests, and the open grey circles represent the ones of the reference soils. Copyright©2019 by Elsevier (reproduced from Chapter II, Lindström et al., 2019. Soil Biology & Biochemistry, in press).

The core indicators of the reference soils mainly suggested associations with plant roots and the rhizosphere. Interestingly, none of the Actinobacterial taxa were frequent and abundant enough in the reference soils to reach core indicator status, whereas two potentially insect-pathogenic fungi, *Pochonia* (Lin et al., 2018) and *Mortierella* (Vega et al., 2012), were mostly absent from the nests but characterized as significantly indicative of the reference soils. This could suggest that the nest represents a hostile environment for these pathogenic fungi, possibly due to the ample presence of Actinobacterial taxa. One taxon, Acidobacteria Gp1 was characterized as indicator species for both the nest and the reference soils, which indicates association with either environment at lower taxonomic levels than those used in our study.

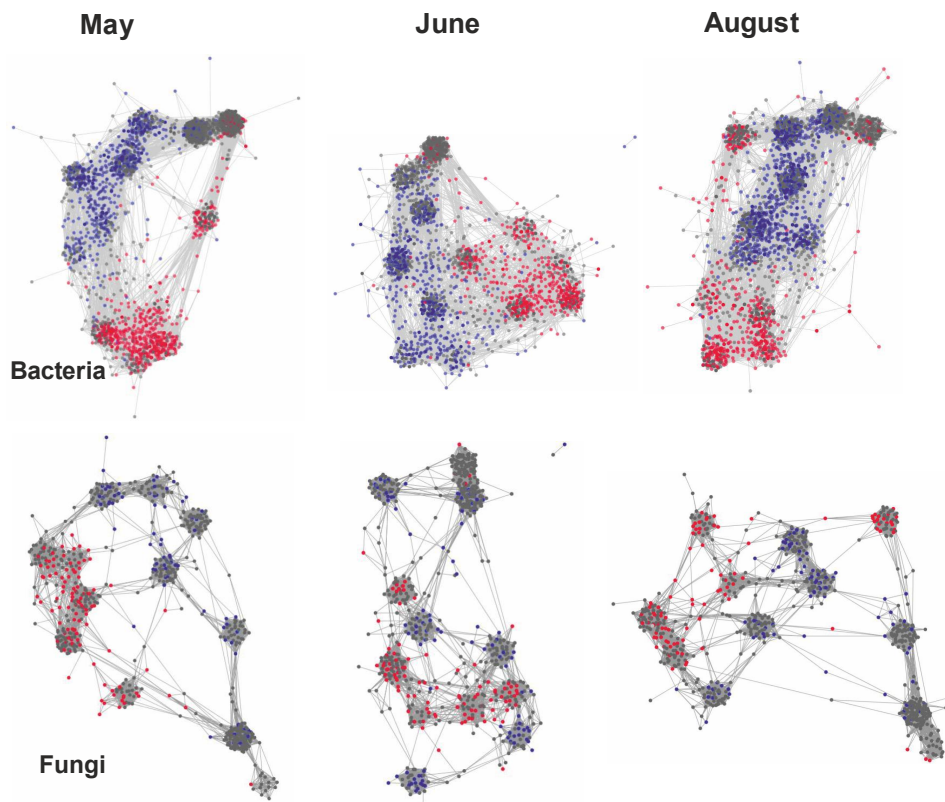


Figure 3. Monthly networks of nests and reference soils, constructed for bacteria and fungi (n=36). Red dots correspond to indicator OTUs in the nest environment, and blue dots to indicator OTUs in the reference soils. All other OTUs are coloured grey. Copyright©2019 by Elsevier (reproduced from Chapter II, Lindström et al., 2019, *Soil Biology & Biochemistry*, in press).

4.3 The temporal stability of nest microbes

The unique nest environment promotes distinct bacterial and fungal communities, which significantly differ from those in the surrounding reference soil. The nest environment characteristics are also inclined to affect the resilience of the microbial communities, manifest as turnover (Hallett et al., 2016) or consistency of taxa within or between seasons. The steady temperature, with shorter annual periods of sub-zero temperature, the differing levels of pH, nutrients and humidity than those in the surrounding soil, together with the nest activities of the ants, all appear to direct the microbes to a divergent trajectory of adaptation than the one in the surrounding soil.

In Chapter III, I demonstrate that the spatial location of the nest explains more variation in bacterial community similarity, than the temporal effects of month and year. In addition, the turnover of the bacterial nest taxa was low, compared to the turnover measured in other studies of soil bacteria (Kavamura et al., 2018), suggesting a strong and consistent bacterial core microbiome. Moreover, this suggests that the variation in the community similarities shown by the PERMANOVA analysis

could be related to species' abundance rather than species composition. During the one-year period, in which I compared the bacterial nest dynamics to the ones in the reference soils, the nest turnover rate was also considerably lower than the one of reference soils, in particular during the period of June to August (Table 3). I found the turnover of the fungal community to be considerably higher than the bacterial one, indicating that a large number of fungal taxa either appeared or disappeared during the sampling period. Based on the T-RFLP data, the similarity of the fungal communities showed inter-annual structures as well. Furthermore, also based on the T-RFLP, the diversity of the bacterial communities varied between the years, but these were not shown in the OTU-subset (results in Chapter III).

Table 3. The species turnover rates and appearance and disappearance rates of the bacterial and fungal nest taxa in 2013–2015, and for the taxa in reference soils 2015. (Reproduced from Chapter III).

	Nest 2013	Nest 2014	Nest 2015	Ref. soil 2015
BACTERIA				
Turnover, proportion (%) of taxa:				
May–June	22	12	18	22
June–August	24	34	17	24
Appearance, proportion (%) of taxa:				
May–June	11	9	8	10
June–August	10	3	6	17
Disappearance, proportion (%) of taxa:				
May–June	10	4	10	13
June–August	14	31	11	6
FUNGI				
Turnover, proportion (%) of taxa:				
May–June	39	31	36	33
June–August	44	40	30	29
Appearance, proportion (%) of taxa:				
May–June	14	18	29	23
June–August	12	12	11	15
Disappearance, proportion (%) of taxa:				
May–June	26	13	8	10
June–August	32	28	20	14

I also identified a temporally consistent set of bacteria and fungi, which was constantly present in all sampled nests, at all sampling occasions, during the three-year sampling period (Chapter III). I identified many of these consistent nest taxa in the reference soil samples as well, but then there was a considerable difference in either their frequency or abundance, or in both, between the two environments. The specific preferences of some taxa, regarding temperature, humidity or nutrient availability, could well explain their consistency, or higher abundance in the nests compared to the

surrounding soils. I detected several taxa, in particular those belonging to Actinobacteria and Proteobacteria, as being both characteristic to the nest environment compared to the reference soils during one season (Chapter II), as well as being temporally stable in the nests, over a three-year period (Chapter III). From an entomological point of view, these two phyla are especially interesting, as they both encompass taxa, which frequently establish symbiotic relationships with ants, (Barke et al., 2010; Ishak et al., 2011; Kautz et al., 2013; Lester et al., 2017; Lucas et al., 2017; Mattoso et al., 2012; Reyes and Cafaro, 2015).

5 Perspectives and conclusions

Unveiling the dynamics and the core members of a community is crucial to understand how a host-manipulated environment, such as a nest, shapes its microbiota. To achieve this, it is essential to tailor a set of robust and reliable methodologies that are specifically adapted to the target community in question. The results of Chapter I show that on its own, the T-RFLP analysis gives a good overview of both the bacterial and fungal communities, although the outcome of the fungal T-RFLP fingerprint was in general somewhat less clear compared to that of the bacterial communities. In support of our hypothesis, the largely similar community patterns gained with both T-RFLP and NGS were convincing throughout the whole project (Chapters I, II and III), confirming that the methods complemented each other. Performing the analyses, which required taxonomic information on NGS data from a limited number of samples, and combining it with community patterns based on a larger data set facilitated a comprehensive analysis on an extensive dataset, while still keeping costs at a moderate level. A major challenge for the taxonomy based analysis, was the lack of sufficiently descriptive and reliable annotations in public DNA repositories, particularly for fungi (Nilsson et al., 2006). Therefore, a rather large part of the microbial OTUs was identified at levels above Class, which provide very little information of the traits and function of the microbe represented by the OTU in question. This of course makes it difficult to assess the overall functionality and reciprocal dependencies of the microbes, or the level of their association with the host and the nest material.

The general outcome of the study on the microbes in the *F. exsecta* nests confirmed our hypothesis, that the bacterial and fungal communities in the nest mounds are unique, compared to the surrounding bulk soil. The island, and the specific nest location also influence the community similarities, but to a much lesser degree. Furthermore, as hypothesized, temporal variation within the nests was negligible compared to spatial variation. The nest communities also showed less monthly variation over one season, appearing seasonally more stable, than the communities in the reference soils. Our study spanned a period of three years, which can be considered relatively long. However, a much longer time span would have been required, to find out about the long-term stability of the microbial communities. For example, does the nest environment protect the microbes from the thermal fluctuations that seem to emanate from climate change.

The variation in the abundances of the taxa, rather than in species identity, explained the main differences between the communities in the nests and the reference soils. The predominant taxa in both environments are commonly found in acidic forest soils. However, we also detected OTUs that were unique to one of the two environments. The most frequent and abundant OTUs were picked up as indicators of their respective environment, but many were excluded from further examination, either because of their low frequency or low abundance. This fraction of the rarer nest taxa certainly

suggests further avenues for study, as it's contribution to community resilience and activity can be disproportionately large (Lynch and Neufeld, 2015; Shade et al., 2014). Members of the "rare biosphere" are often ecologically important, some becoming periodically abundant when suitable circumstances occur, and some being permanently rare, while still playing ecologically critical roles (Lynch and Neufeld, 2015).

Identifying a set of environment-characteristic indicator taxa rendered further support for the hypothesis of distinct nest microbes. Characterizing the core members of a community, is crucial, in order to understand which taxa are of pivotal importance for ecosystem functions. The most characteristic and the most consistent taxa in the nests indicated patterns of association either with the nest building material, or with the ants themselves. The co-existence of the social and genetically uniform ants and a consistent and stable microbiome is expected to endorse co-evolution, possibly on a relatively fast rate. A gut microbiome evolves within one host, whereas the ant nest microbiome interacts with all colony members. It also raises the question of how vital the microbes are to the ants – in our study several taxa characteristic to the nest belonged to the phylum of Actinobacteria, such as *Pseudonocardia* or *Streptomyces*, both known to produce antibiotic substances (Engl et al., 2018). Is the presence of beneficial Actinobacteria required, for the nests to stay active up to 30 years, as many *F. exsecta* nest do (Pamilo, 1991)?

Except for a number of studies performed on leaf-cutting ants, microbes in ant nests are largely uncharted, particularly regarding their variation in time and place, or in comparison with the surrounding soils. Overall, our results show that the rigorously maintained and curated *F. exsecta* nest environment hosts unique and stable bacterial and fungal communities. The ants seem to have a considerable influence on the composition and the stability of the microbial communities in their nests. The communities encompass several taxa, which based on their functional traits are of great ecological interest. Our results contribute to our understanding of microbiomes in patchy habitats, and open up for further studies on the dynamics of microbial communities in secluded environments.

The obvious next step in the research of ant nest characteristic microbiota would be to expand the assessment of microbes to those in the gut, haemolymph and cuticle of ants, in order to investigate the level and identity of any symbiotic associations. In particular, investigating the antibiotic-producing Actinobacterial taxa and their role in the disease resistance of ant colonies would be an interesting path to explore, as it would contribute to our knowledge of resistance mechanisms in insects. Furthermore, it could provide valuable insights into antibiotic-producing microbes that obey the selective forces prevailing in a distinct environment. Moreover, in this work, I have studied bacteria and fungi in parallel, and along the way, caught some exiting glimpses of potential associations between the members of these two kingdoms. Studying the possible interactions between bacteria and fungi, associated with ants and their nests, would render comprehensive knowledge of a multi-layer, ecological system.

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