The role of small mammals as vectors for spores of ectomycorrhizal fungi in Central European mountain forests

Kumulierte Dissertation

vorgelegt von

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Vorwort

Wälder sind komplexe Gefüge mit einer Vielzahl von wenig bekannten ineinandergreifenden Prozessen. Für eine nachhaltige Nutzung und eine Bewahrung der Biodiversität und Funktionalität, müssen wir alles daran setzen diese multifaktoriellen Wechselwirkungen zu verstehen. Nur wenn wir in der Lage sind, die Funktionalität zu erhalten, werden wir und auch nachfolgende Generationen weiterhin die Vielzahl an materiellen Rohstoffen und immateriellen Werten nutzen und genießen können.

Es finden sich jedoch noch viele Lücken im Wissen über diese Zusammenhänge in Wäldern. Im Bestreben einen Anteil zu leisten, sie zu verkleinern, entwickelten Prof. Klaus Hackländer, Dr. Ursula Nopp-Mayr und Dr. Alexander Urban mit mir ein Forschungsprojekt, welches auf verschiedene Weisen die Rolle von Kleinsäugern im Hinblick auf die Verbreitung von Pilzsporen in alpinen Bergwäldern beleuchten sollte.

Die Funktion von Kleinsäugern in Wäldern ist eine der unsichtbaren, faszinierenden Interaktionen zwischen Waldbäumen, Mykorrhizapilzen und Tieren. Während die Bäume und die Mykorrhizapilze in einer engen Symbiose miteinander leben und beide Partner vom jeweils anderen hinsichtlich Wachstum und Nährstoff- bzw. Wasserversorgung profitieren (wood wide web), hat sich auch zwischen den Mykorrhizapilzen und den ihre Fruchtkörper fressenden Tieren eine mutualistische Beziehung herausgebildet (Abb. 1).
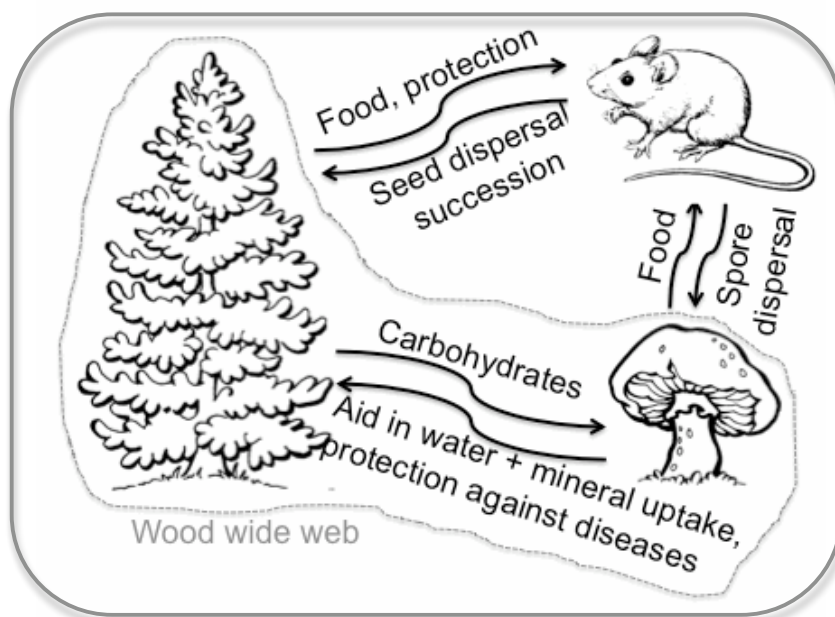

Fig. 1: Verwobene Beziehungen im Wald / Interweaving relationships in the forest

Viele der Mykorrhizapilze, die mit Waldbäumen in gemäßigten Zonen Symbiosen eingehen , bilden sind ektomykorrhizale (ECM) Symbiosepartner von u. a. *Pinus*, *Picea*, *Abies*, *Quercus*, *Fagus* und bilden charakteristische ober- oder unterirdische Fruchtkörper (Ständerpilze oder Trüffel). Da die Fruchtkörper der hypogäischen ECM-Pilze die Mechanismen der selbstständigen Sporenabgabe verloren haben, die bei oberirdisch fruchtenden Pilzen eine Sporenverbreitung mit Wind oder Wasser gewährleisten, sind sie auf endozoochorische Verbreitung der Sporen angewiesen. Außerdem lässt die Phylogenie dieser Pilze eine wiederholte Entstehung der unterirdischen Fruchtkörperbildung vermuten, so dass die Sporenverbreitung durch Tiere (Endozoochorie) erfolgversprechend zu sein und zuverlässig zu funktionieren scheint. Mycophagen Tieren (Kleinsäuger und andere Säuger bzw. Beuteltiere, aber auch Wirbellose) kommt daher für die Verbreitung von trüffelähnlichen Pilzen besondere Bedeutung zu.

Kleinsäuger verbreiten nicht nur die einzelnen Pilzarten und sorgen so für eine Erhöhung bzw. Erhaltung der Biodiversität, sondern sie transferieren auch die keimfähigen Sporen in kleinen Nährstoffpaketen zu geeigneten Habitaten in denen sie leicht mit Samen und Keimlingen von Bäumen in Kontakt kommen können. So tragen Kleinsäuger auch zur (Re-)Kolonialisierung der Mykorrhizapilze in bestimmten Gebieten bei. Den tierischen Konsumenten der Pilze kommt also nicht nur die Rolle des "Säenden" zu, sie beteiligen sich auch an der Bewahrung der Stabilität des gesamten Systems, da sie die Ausbreitung und Aufrechterhaltung des sogenannten "wood wide web" gewährleisten.

Nun gibt es viele Tierarten die sich gelegentlich, teilweise oder sogar ausschließlich von Pilzen ernähren, aber ihre Rolle und Wichtigkeit für das gesamte System Wald ist erst in zwei großen Gebieten der Erde detaillierter untersucht worden. Für Mitteleuropa, und besonders für die alpine Region, gibt es nur wenige Forschungsergebnisse, obwohl gerade hier die Funktionalität der Waldgebiete essentiell im Hinblick auf Schutzfunktionen (gegen z. B. Lawinen, Muren, Steinschläge oder Überschwemmungen) und weitere ökonomische sowie soziale Werte ist.

Dieser Wissensmangel war ein wesentlicher Grund für die Erarbeitung und Durchführung des vorliegenden Projektes und der damit verbundenen Dissertation.

Meinen drei Betreuern bin ich zu großem Dank verpflichtet, denn durch die kompetente Anleitung und profundes Wissen wurde das Projekt erfolgreich genehmigt und gefördert. Während der gesamten Zeit standen sie mir stets mit Rat und Tat zur Seite und sind

Introduction

Forests are far more complicated than most people think and only if scientists and practitioners thrive to understand the complexities and interdependencies of species and habitats they can develop sustainable ecosystem policies. Sustainable forest management is crucial if we want to keep forests resilient and functioning for us and for further generations and enjoy all of their ecological, economic and social values.

One of the multiple interactions taking place constantly in a forest is the one between trees, mycorrhizae forming fungi and mycophagous animals (Fig. 1).

The mycorrhizal fungi live in a mutualistic relationship with their host trees, forming a tightly knitted network of very fine hyphae within and between the feeder rootlets of forest trees. By this symbiotic life they enhance water and mineral uptake of the trees and are provided with essential carbohydrates in return (Smith and Read 1997). Also, there is evidence, that the symbiosis with mycorrhizal fungi gives the trees some protection against diseases and heavy metal pollution (Galli et al. 1994; Azcón-Aguilar and Barea 1997; Branzanti et al. 1999). And even further, Simard et al. (1997) showed, that the fungal hyphae of the mycorrhizae connect different trees of the same and of different species within the forest and form the so called wood wide web. This web spans entire temperate forests and interconnects various tree and fungal species (Simard et al. 1997). In temperate forests most tree species form mycorrhizae with ectomycorrhizal (ECM) fungi, which do not penetrate the root cells of the trees, but rather form the so called Hartig net in the intercellular space and envelop the root tips in sheaths of fungal hyphae (Smith and Read 1997). These fungal

species often belong to the truffle like species, maybe because hypogeous fruiting is more suitable in the highly seasonal climate pattern of temperate regions. Fruiting below ground has the major advantage of being independent to a certain extent from severe weather conditions such as drought or frost, so there is evidence, that this fruiting habit evolved independently in several ECM fungal taxa (Johnson 1996). Furthermore, many of them (either hypogeously or epigeously fruiting species) are desirable delicacies.

But, for distribution of seeds and spores as well as subsequent rejuvenation and (re)colonisation both trees and fungi rely on third party help. But while lightweight tree seeds and spores of epigeously fruiting (mushroom like) fungi are dispersed by wind and, to a lesser extent, water, hypogeously fruiting (truffle like) fungi and heavy tree seeds depend on other vectors. To a large extent this vector function is taken over by animals. Mycophagy (the use of fungal fruit bodies for nutrition) and frugivory (in case of forest trees often called seed predation) is a common feeding habit in many animal species, both of the invertebrate and vertebrate kind. It is essential for spore dispersal of truffles, as their fruit bodies not only lie beneath the surface, but also have lost mechanisms of forcible spore discharge (Johnson 1996).

Many animal species have been found to feed on fungal fruit bodies and subsequently act as spore dispersers, including marsupials, small mammals, deer, elephants, turtles, beetle larvae or even birds (Fogel and Trappe 1978; Ure and Maser 1982; Launchbaugh and Urness 1992; Claridge and Lindenmayer 1998; Simpson 2000; Claridge et al. 2001; Paugy et al. 2004; Harrington 2005; Ashkannejhad and Horton 2006; Jones et al. 2007).

This interrelationship has been studied in detail in two regions of the world – the Pacific Northwestern United States and Southeastern mainland Australia. There, many mammals and marsupials (including Californian red-backed vole, Northern flying squirrel, Douglas squirrel, Pacific jumping mouse, Long-footed potoroo, Swamp wallaby, Bush rat, Eastern pygmy-possum) have been shown to be mycophagous to some extent, using mostly truffle like fungi for nutrition (Maser et al. 2008).

But, research into different species communities in other temperate regions of the world remains scattered. Some attempts were made in Europe (Blaschke and Bäumler 1989; Bertolino et al. 2004), but so far there was no investigation into the mycophagy of a small mammal community in Central European mountain forests. However, investigations would be very important as we cannot compare different sets of species from other temperate

regions of the world with the species complex encountered in Central Europe. This lack of knowledge is regrettable, because of the role a resilient and healthy forest plays in Central European mountain ranges. Not only is the forest used for economic reasons and recreational purposes, it also forms a major protection against erosion, avalanches, mud slides and floods (Brang 2001). The value of an intact and functioning wood wide web sustaining a healthy and resilient forest can thus not be overestimated.

We therefore set out to evaluate the vector potential for ECM fungal spores of the small mammal species forming the Central European mountain forest community. The interweaving process of mycophagous animals feeding on fungal fruit bodies (sporocarps), dispersing the spores and formation of mycorrhizae requires an interdisciplinary approach. So, we used multiple ways to evaluate the vector function of small mammals (Fig. 2).
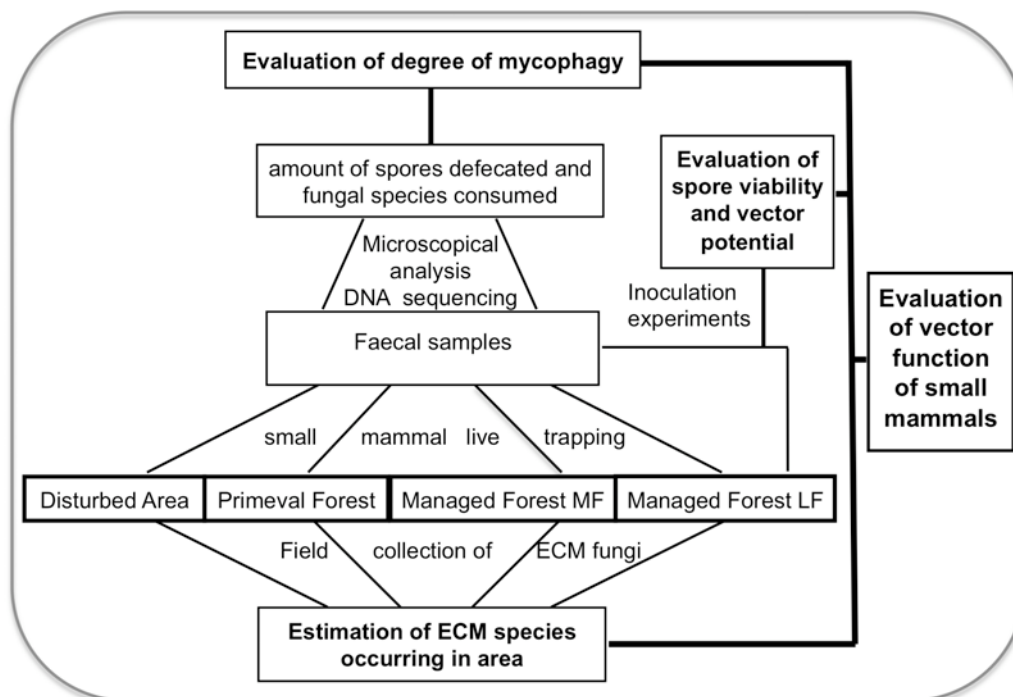
Fig. 2: Interdisciplinary and multi-step project layout

Besides the direct evaluation of small mammal mycophagy with microscopical and DNA based fungal spore determination from faecal samples, we used greenhouse and field inoculation experiments to assess the vector potential of the single small mammal species. As we also had the opportunity to investigate different forest types, we were able to consider management differences regarding small mammal community composition, fungal species occurrence, degree of mycophagy and small mammal vector functions. By this

multidimensional approach we could not only take different trophic levels into account, but could also investigate the structure of the fungi – mycophagist bipartite network.

As the structure and organisation of mutualistic bipartite networks has received renewed interest in recent years (Bascompte and Jordano 2006, 2007; Montoya et al. 2006), the structure of the relationship between fungi and their consumers might add to considerations about stability and persistence of communities (Fortuna et al. 2010).

Various indices for characterisation of network properties have been proposed and evaluated (Dormann et al. 2009; Blüthgen 2010), so there are many possibilities to assess the given bipartite relationship. As we have interaction frequency data for our network we turned the quantitative indices and attempt the first comparison of mutualistic mycophagy webs of different ecosystems.

Methodology

1. Survey plots

As forest management or disturbances seem to have an impact on small mammal community structure, degree of mycophagy and on sporocarp production (Keinath and Hayward 2003; Luoma et al. 2004; Jacobs and Luoma 2008), we took the unique opportunity to compare the largest remnant of an old-growth (never managed) forest in Central Europe with a managed forest and a large disturbed area (wind throw in 1990) in close proximity. This was done in the Dürrenstein Wilderness Area (DWA, 47° 48' to 47° 45' N, 15° 01' to 15° 07' E, 2300 ha) in the Northeastern Limestone Alps (Austria), where there are about 400 ha of primeval forest (Zukrigl et al. 1963) surrounded by sustainably managed forest and some naturally disturbed areas. For management comparisons and for the possibility to carry out inoculation experiments in the field, we chose another managed forest area of similar tree species composition in the Rosalia Mountains on the eastern margin of the alps (Austria). This survey area was situated in the Rosalia Demonstration Forest of the University of Natural Resources and Life Sciences Vienna (RDF; 47° 42' 0'' N, 16° 17' 52'' E, 930 ha). For a detailed description of the four investigated forest areas refer to chapter 1 of the Results and Figures 3 to 6.

Fig. 3: Wind thrown survey plot (DWA)   Fig. 4: Primeval forest (DWA)

Fig. 5: Managed forest survey plot (DWA)   Fig. 6: Managed forest survey plot (RDF)

2. Small mammal trapping

We conducted a small mammal live trapping survey with five trapping sessions (July and October 2006 and June, August, October 2007) in the Dürrenstein Wilderness Area (DWA) and eight sessions (April, July, September, January 2006 and 2007) in the Rosalia Demonstration Forest. We trapped for three consecutive nights according to international standards (Kirkland Jr. 1998; Powell and Proulx 2003) on two fixed grids per survey plot with a total of 50 small mammal live traps per grid. This results in a total of 4500 trap nights in the Dürrenstein Wilderness Area and in a total of 2400 trap nights in the Rosalia Demonstration Forest. The four forest areas were primeval forest (DWA), managed forest 1 (DWA), managed forest 2 (RDF), and disturbed area (DWA; wind throw).

We used trapping grids with a five by five trap station array, with two traps at each station and stations spaced 15 m apart. The outer gridlines were at least 100 m apart from any

different adjacent forest types and the trapping plots were between 300 and 3000 m apart from each other to minimize chances of crossing small mammals.

We applied two different kinds of small mammal live traps (Field Trip Traps and Wooden Box Traps) containing peanut butter cookies and apple slices as bait as well as mealworms, rodent chow and hay for food and thermal cover (Field Trip Traps only) during the survey, to estimate trap type influence on catchability.

We transferred captured animals from traps into clear plastic bags, identified them by using morphological traits (Corbet et al. 1982), and weighted, measured, and sexed each animal. We applied a recapture identification, and set the animals free at the place of capture. After taking faecal samples we thoroughly cleaned each trap, replenished bait, food, and insulation and reset the trap in the same spot.

3. Sampling procedure and sample storage

For the faecal samples we collected the scats left in the traps. We sampled each animal only once to reduce bias by trap happy animals. We stored samples in Eppendorf reaction tubes (1.5 ml) filled half with silica gel beads for desiccation. Once dry, we separated pellets from coarse plant material as well as from silica beads, added 600 µL distilled water and homogenized them mechanically with a conical pistil in the Eppendorf reaction tubes. We stored the resuspended samples at -20 °C for further analyses.

4. Microscopical analyses

We transferred two drops of the thoroughly homogenised sample solution with sterile broad bladed forceps onto a microscopic slide, setting one drop apart from the other. We then added one drop of Melzer's reagent (Morton 1989) to one of the subsamples in order to stain the polysaccharide components of the fungal spores. For the other subsample we used Hoyer's mounting medium (Cunningham 1972) as a fixation solution. Finally we homogenised both drops thoroughly using a preparation needle, covered them with a cover slip and sealed each subsample with nail polish to prevent evaporation.

We used a Reichert Polyvar light microscope with 100 to 1000fold magnification and custom fitted with a NIKON digital camera. We analysed each sample separately, selecting 25 random fields of view (fov) with 400fold magnification for each of the two subsamples per slide. We counted all spores visible in the field of view, classified each spore type and produced a detailed description of each spore type with 1000fold magnification, including

parameters like size, shape, colour stained and unstained, ornamentation and occurrence of a suprahilar plague and sterigmal remnant.
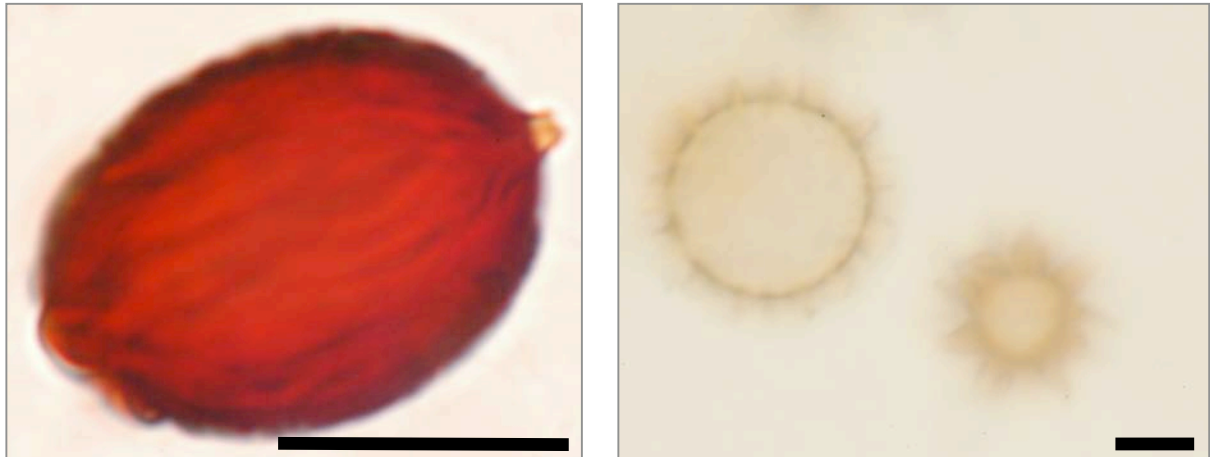

Fig. 7: Stained spore of *Chamonixia caespitosa* (left, 1000x magnification) and unstained spores of *Tuber rufum* (middle, 400x) and *Octaviania asterosperma* (right, 400x), scale bar = 10 µm

5. Molecular analysis

First, we established a reliable and easy to use DNA extraction protocol and evaluated it in comparison with other protocols (see chapter 2 of the Results). After establishing the protocol we extracted DNA from more than 100 samples to obtain as many fungal DNA sequences as possible.

As direct sequencing failed in many samples due to high heterogeneity (samples contained spores of different types in varying amounts), we also applied cloning and pyro-sequencing to enhance results.

6. Field collection of fungal fruit bodies

For direct comparison of spore morphology and DNA sequences as well as for an estimation of the availability and abundance of fungi the mycologists of the Mycology Research Group, University of Vienna collected fruit bodies in both survey areas.

7. Inoculation experiments

a. Green house experiments 1

Inoculation experiments started with the onset of the growing season in spring 2007. We used beech, spruce, and fir seeds obtained from a commercial nursery (HERZOG.BAUM Samen und Pflanzen GmbH, Gmunden, AT), which were surface sterilized with hydrogen peroxide (10 %, 10 min) stratified and germinated in the cool room at about 8° C. After germination seedlings were individually potted in multi pot plates (M35, 96 individual pots,

35 mm diameter) filled with heat sterilised substrate (2 h, 65° C, consisting of one part each of Perlite, sand and unfertilised nursery substrate). Immediately after potting we added a solution of faecal material (0.5 ml / pot) of one of the four main small mammal species and from one of the investigated forest types. Due to the restricted amount of faecal material obtained during the 2006 live trapping, we could not use a higher amount of faecal suspension and inoculated between ten and twenty seedlings per tree species, small mammal species and forest plot.

Harvest of the seedlings took place in late autumn 2007 and feeder rootlets of the seedlings were microscopically analysed for presence and type of mycorrhizae formed.

b. Green house experiments 2

To test the effect of root exudates of arbuscular mycorrhizal host or non-host plants on the germination of a certain parasitic plants, exudates of a range of plants were used for germination stimulation experiments. See chapter 3 of the Results for details.

c. Field experiments

In the Rosalia Demonstration Forest we randomly selected 30 places to bury large plastic boxes (lbh 45/30/30, with µm-mesh covered drainage holes) filled with heat sterilised substrate (same as in greenhouse inoculation trials). Twenty boxes were then covered with mesh cages fitting closely (height 30 cm) of one of two mesh widths (10 mm preventing small mammal access and 25 mm allowing it). Ten boxes were left uncovered and all boxes were left to settle and integrate with the environment during winter. With the onset of the vegetation period 2007 ten tree seedlings of each of the three species as for the greenhouse inoculation experiments (taken from the same stock) were planted into the boxes in random places and left to grow for the entire season. After harvest of the seedlings in late autumn 2007 feeder rootlets were analysed similarly to the greenhouse seedlings.

The field inoculation experiment was repeated in 2008 with partly sterile and partly natural seedlings.

8. Calculations and Statistics

Due to the heterogeneity of the resulting data we had to use different statistical methods for evaluation of the specific data sets (small mammal capture results, evaluation of

microscopic spore identification and count, DNA extraction comparison, comparison of microscopic and molecular determination of fungal spore types).

Small mammal capture data were analysed using non parametric tests like Kruskal-Wallis-Test and post hoc comparison established by Sokal and Rohlf (1995) and described by Gardener (2006). For further evaluation of small mammal capture results we applied a spatially explicit capture recapture analysis (secr) (Nopp-Mayr *et* al. in prep.).

We examined potential differences in DNA extraction efficiency between the three tested protocols applying a one-way ANOVA with repeated measurements and a two-way ANOVA with spore number as co-factor to account for potential correlation between number of fungal spores in the samples and PCR efficiency with fungus-specific primers (see chapter 2 of the results).

For evaluation of small mammal mycophagy we used a variety of approaches suitable for the specific subset of data. We applied Kruskal-Wallis-Tests to investigate overall differences in number of ingested ECM spores between small mammal species and used negative binomial regression to evaluate differences in number of ECM spores per sample regarding small mammal species, collection session and forest plot. To determine differences in number of ECM species found in single samples we used MANOVA and TUKEY multiple comparison of means after square root transformation of the dataset.

We furthermore calculated network indices of our small mammal – ECM fungi food web using the bipartite package of R (Dormann et al. 2008) and compared it with different mycophagy data sets from various ecosystems.

Due to small sample sizes results from the field collection of fungi and the inoculation experiments were evaluated qualitatively only.

Results
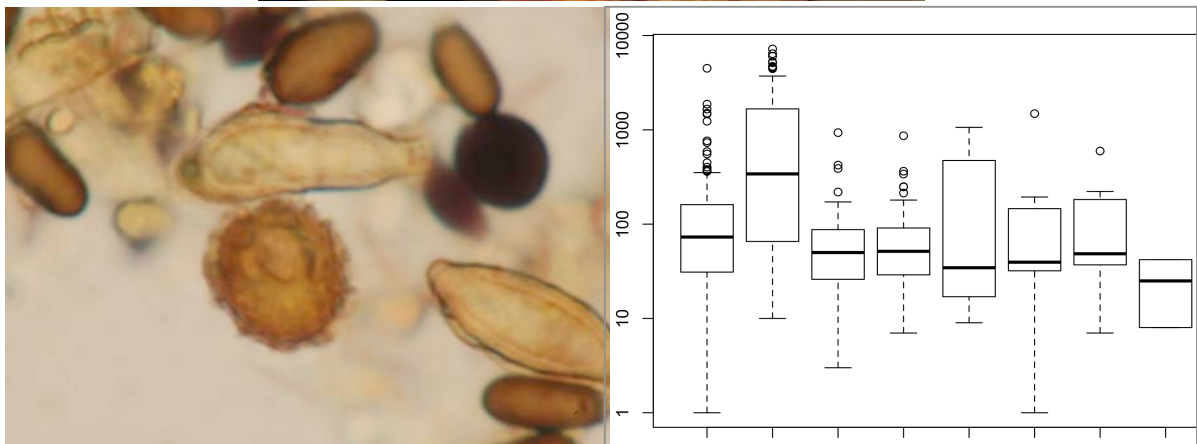
From top left: Active small mammal live trap, *Myodes glareolus* – main mycophagist, King bolete (*Boletus edulis*) with rodent tooth marks, Fungal spores under microscope, Number of defecated spores of small mammal species

1. *Small mammal mycophagy*

The interrelationship of mycophagous small mammals and ectomycorrhizal fungi in primeval, disturbed and managed Central European mountainous forests

Spores of *Octaviania asterosperma* and *Melanogaster broomeianus*, scale bar = 5 µm (left) and *Myodes glareolus* feeding on fungus (right)

The interrelationship of mycophagous small mammals and ectomycorrhizal fungi in primeval, disturbed and managed Central European mountainous forests

1   **The interrelationship of mycophagous small mammals and ectomycorrhizal fungi in primeval,**

2   **disturbed and managed Central European mountainous forests**

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14   **Abstract**

15   Small forest dwelling mammals are considered to be major consumers and vectors of hypogeous

16   ectomycorrhizal (ECM) fungi, which have lost the ability of active spore discharge. Fungal spore

17   dispersal by mycophagy is deemed an important process involved in forest regeneration, resilience and

18   vitality, primarily based on evidence from Australia and the Pacific Northwestern USA, but is poorly

19   known for Central European mountainous forests thus far.

20   Small mammal mycophagy was investigated by live trapping and microscopical analysis of faecal

21   samples. All small mammal species recorded (*Myodes glareolus*, *Microtus agrestis*, *Pitymys*

22   *subterraneus*, *Apodemus spp.*, *Glis glis*, *Sorex* spp.) had ingested spores of ECM fungi, albeit in

23   varying amounts. *My. glareolus* was found to be the most important vector of ECM fungal spores,

24   both in quantity and diversity. Species of the genus *Sorex* seem to play a hitherto underestimated role

25   as dispersers of fungal spores. *Glis glis* is likely to be an important vector due to its large home range.

[1] SS, AU, KK designed and carried out experiments, analysed and evaluated results and wrote the
manuscript
AU, UNM, KH developed original ideas, evaluated results and edited the manuscript

26   Hypogeous ECM basidiomycetes accounted for most spores found in the faecal samples. The

27   frequency of various genera of hypogeous ECM ascomycetes and ECM epigeous fungi was much

28   lower. Comparison with null models indicated a non-random structure of the mycophagy network

29   similar to other mutualistic bipartite networks. Mycophagy can be considered (1) to contribute to

30   nutrition of small forest mammals (2) to play a pivotal role for forest regeneration and functioning by

31   providing mycorrhizal inoculum to tree seedlings and (3) to be vital for reproduction and diversity of

32   the still poorly known hypogeous fungi.

33   **Keywords:** rodents, shrews, truffles, mutualism, nestedness

34   **Introduction**

35   Mycophagy, the use of fungi as food source, is recognised as a feeding habit of many animal species

36   in different ecosystems as well as a major way of spore dispersal for hypogeous fungi, which almost

37   exclusively form ectomycorrhizae (ECM) (Fogel and Trappe 1978; Johnson 1996; Vernes and Dunn

38   2009). Hypogeous fungi lack mechanisms of spore discharge to the air (Fogel and Trappe 1978) and

39   reconstructions of fungal phylogenies suggest that the hypogeous fruiting habit evolved repeatedly

40   from epigeously fruiting genera in ascomycetes and basidiomycetes (Johnson 1996; Trappe and

41   Claridge 2005). This implies that endozoochory is a successful way of spore dispersal. Higher

42   independence from weather conditions, the pelleting of spores in nutrient reserves attractive for fine

43   roots and the visiting of favourable habitats by fungivorous mammals are the major advantages of this

44   tripartite mutualism involving mycorrhizal plants, mycorrhizal fungi and fungivorous animals

45   (Johnson 1996). In turn, mycophagists can indirectly influence vegetation succession by dispersing

46   propagules of mycorrhizal fungi, thereby promoting the spread and regeneration of obligate

47   mycorrhizal plant (Bruns 1995; Terwilliger and Pastor 1999; Wiemken and Boller 2006). Maser et al.

48   (2008) hypothesize, that without animal dispersal of spores of hypogeous fungi, growth, regeneration

49   and adaptation of the mycorrhizal fungi-tree-network would be greatly impaired if not impossible.

50   Many animal species in different ecosystems have been shown to be mycophagous (Ure and Maser

51   1982; Claridge and Lindenmayer 1998; Tuno 1998; Paugy et al. 2004; Jones et al. 2007). Maser et al.

52   (2008) classify mycophagists as obligate (e. g. *Myodes californicus*, *Potorous longipes*); preferential

53   (e. g. *Glaucomys sabrinus*, *Bettongia penicillata*); opportunistic (e. g. *Peromyscus sp.*, *Oreamnos*

54   *americanus*, *Alces alces*, *Wallabia bicolor*) or accidental mycophagists (e. g. predators of

55   mycophagists, *Dasyurus sp.*, *Striiformes*) according to their degree of mycophagy.

56   Even though there are some data available from other biogeographic regions (e. g. Reddel et al. 1997;

57   Mangan and Adler 2002), the majority of studies have been undertaken in the Pacific Northwestern

58   USA and in Australia. For these two regions research has proven the pivotal role of mycophagy for

59   both animal conservation and reproduction of a diverse range of hypogeous fungi (Cázares and Trappe

60   1994; Frank et al. 2006; Vernes and Dunn 2009); and has already lead to management implications

61   (Carey et al. 2002; Dell 2002; Wiensczyk et al. 2002).

62   But, are the results achieved and the conclusions drawn also applicable to Central European

63   mountainous forests with their specific plant, animal and fungal communities?

64   A few studies provide first insights: Drożdż (1966) observed that *M. glareolus* turns to mycophagy

65   when beech seeds are sparse. Blaschke and Bäumler (1989) investigated mycophagy and spore

66   dispersal of some small mammal species in Bavarian forests but did not analyse in detail the fungal

67   species consumed. Grönwall and Pehrson (1984) and Bertolino et al. (2004) studied mycophagy of the

68   red squirrel, and Wiemken and Boller (2006) investigated the role of ungulates as mycophagists. One

69   very recent study from Lithuania (Kataržytė and Kutorga 2011) evaluated the degree of mycophagy of

70   various small mammals in different forest types: They found, that *Myodes glareolus* exhibited the

71   highest numbers and diversity of fungal spores in faecal samples and that faeces of shrews contained

72   more fungal spores than previously guessed.

73   Among the open questions related to mycophagy, the influence of natural disturbances and forest

74   management practices on the community structures of both small mammals and ECM fungi, and on

75   the degree of mycophagy (Carey et al. 2002; Jacobs and Luoma 2008) is still unknown in Central

76   European mountainous forests. In primeval or sustainably managed forests, ECM occurrence is

77   unlikely to limit tree growth or mycophagy due to the typically high levels of mycorrhizal colonisation

78   and ECM fungal diversity (Luoma et al. 2004). Conditions approximating primary succession (large

79   scale wind throws, avalanches, fires, floods, or large scale forest replacement by human activities) on

80   the contrary might lead to the disruption of the mycorrhizal network (Perry et al. 1987). In such

81   conditions, the recolonisation by trees might be limited by the availability of ECM fungi and aided by

82   supply of germinable ECM spores through defecation (Cázares and Trappe 1994; Terwilliger and

83   Pastor 1999; Wiemken and Boller 2006). To achieve a more complete understanding of regeneration

84   and colonisation processes in forest ecosystems and adjacent areas, the vector function of animals in

85   dispersal of ECM spores needs to be evaluated.

86   The specific structure of mycophagist-fungus interaction networks seems to be an open question, too.

87   The relationship between mycophagists and fungi might be highly nested in ways that are known from

88   plant-pollinator or plant-frugivore food webs (Bascompte et al. 2003), but could also be random,

89   dependent only on fungal availability. Nested arrangement of food webs means that there is a core of

90   species in both trophic levels interacting with a larger number of members of the other trophic level

91   along with a number of more peripheral species interacting with a subset only. The specific structure

92   of mutualistic networks influences species dispersal, persistence and coexistence (Bascompte et al.

93   2003).

94   To answer some of the pending questions regarding the mycophagist-fungi-network we focus on four

95   hypotheses regarding small mammal mycophagy and the fungal species consumed:

96   a. Central European small mammals inhabiting forested ecosystems show differences regarding

97   degree of mycophagy and fungal species consumed.

98   b. Degree of mycophagy and fungal species consumed show seasonal and yearly variation.

99   c. Forest area (microhabitat) has an influence on degree of mycophagy and fungal species

100   consumed.

101   d. The mycophagist-fungi-interrelationship is non-random.

102   **Materials and Methods**

103   Survey area and period

104   We collected small mammal faecal material in the Dürrenstein Wilderness Area, Austria (47° 48' to

105   47° 45' N, 15° 01' to 15° 07' E, 2300 ha) and in the Rosalia Demonstration Forest, Austria (47° 42' 0"

106   N, 16° 17' 52" E, 930 ha). Within the Dürrenstein Wilderness Area six survey plots (each about 0.8 ha)

107   were studied, two each situated in Austria's largest primeval forest (PF), in an adjacent managed forest

108   (MF1) and in a disturbed area (DA; wind-thrown in 1990). A further two survey plots were studied in

109   managed forest (MF2) in the Rosalia Demonstration Forest.

110   The Dürrenstein Wilderness Area is located in the eastern part of the Northern Limestone Alps. The

111   climate is suboceanic – subcontinental with long winters and short, cool summers. Annual

112   precipitation reaches about 2000 mm with peaks during summer and winter months. Deep and wet

113   snow cover is lasting, shortening the growing season (Splechtna et al. 2005).

114   The two PF plots were located in the primeval forest found in a watershed on the southern slopes of

115   the Dürrenstein mountain (900-1200 m a.s.l.). The forest vegetation is classified as Asperulo-Abieti-

116   Fagetum and Adenostylo glabrae-Abieti-Fagetum (Splechtna et al. 2005). European beech (*Fagus*

117   *sylvatica*) is the dominating tree species in the primeval forest, but the co-dominant European silver fir

118   (*Abies alba*) and Norway spruce (*Picea abies*) grow 10 to 15 m taller, thus forming a two-layered

119   canopy (Zukrigl et al. 1963). The amount of snags and downed coarse woody debris was estimated at

120   82.6 m^3ha^{-1} and 134.2 m^3ha^{-1}, respectively (Gratzer G., pers. comm.).

121   The two managed forest plots (MF1) were situated in spruce-dominated spruce-fir-beech forest. The

122   distance to parts of the primeval forest was small, so climatic and geological parameters were the

123   same, as was the potential natural vegetation. Contrary to the PF the mature trees were of rather

124   uniform size and spacing. Due to harvest activities the canopy was recently thinned, and we found

125   higher cover of ground vegetation (tree regeneration, *Vaccinium myrtillus*, graminoids, ferns and

126   mosses), abundant small woody debris and a limited amount of coarse woody debris.

127   The two disturbed area plots (DA) were situated on the southern slope of the Dürrenstein mountain.

128   The general climatic and geological features, as well as the potential natural vegetation were the same

129   as for the primeval and managed forest plots, but microclimatic conditions differed due to loss of tree

130   canopy and southern exposure of the site. We observed a locally dense cover of ground vegetation

131   (graminoid, herb, and perennial shrub species) and patches of dense tree regrowth dominated by beech

132   or, more rarely, by spruce, with interspersed maple (*Acer pseudoplatanus*) or rowan (*Sorbus*

133   *aucuparia*) in a tessellate pattern. Also, we encountered extraordinarily high amounts of coarse woody

134   debris in different stages of decay as a result of the wind throw.

135   The other two investigation plots (MF2) were situated in the Rosalia Demonstration Forest in the

136   Rosalia Mountains, at the north-eastern margin of the Alps. Reaching from 350 to 750 m a.s.l., the

137   area is characterized by moderate winters, warm summers and an average annual precipitation of 800

138   mm. There are beech-dominated forest stands as well as spruce-fir-beech forests at higher elevations

139   and northern slopes (Marschall and Sagl 1986). The investigated forest plots (MF2) were located

140   between 600 and 700 m a.s.l. and characterised by spruce-fir-beech forest with an understory mainly

141   consisting of beech regeneration and various graminoids, ferns and perennial shrubs (*Rubus* spp.,

142   *Atropa belladonna*). There were no signs of recent logging, small woody debris and a low amount of

143   coarse woody debris were present throughout the plots.

144   We live trapped small mammals according to international standards (Gannon and Sikes 2007) on all

145   eight plots in summer and autumn 2006 and 2007, resulting in four trapping sessions. We sampled

146   every plot once for three consecutive nights per trapping session.

147   Trapping and sampling procedure

148   We arrayed traps in a five by five grid, spaced 15 x 15 m and placed two traps at every station. Traps

149   were equipped with peanut butter cookies, apple slices, rodent chow, mealworms, and hay as bait,

150   food, and thermal insulation. After each capture we thoroughly cleaned the traps, refilled and reset

151   them in the same spot. We identified animals based on morphological traits (Corbet et al. 1982) and

152   marked them for recapture recognition. Permission and method approval was obtained from the

153   administrations concerned prior to trapping.

154   From each newly captured animal we took faecal pellets from the trap. We sampled each animal once

155   only and stored samples in Eppendorf reaction tubes (1.5 ml) filled with 1 ml silica gel beads as

156   desiccant.

157   Microscopical analysis

158   We separated dry pellets from debris and silica gel beads, then suspended and macerated each sample

159   in 600 µl distilled water, and kept samples frozen at -20°C for further use.

160   We transferred two drops of the thoroughly homogenised sample solution with broad bladed forceps

161   onto a microscopic slide, setting the drops apart from another. We then added one drop of Melzer's

162   reagent (Morton 1989) to one subsample to stain the polysaccharide components of the fungal spores.

163   We mounted the other subsample with Hoyer's mounting medium (Cunningham 1972). After separate

164   homogenisation with a preparation needle, we covered subsamples with a cover slip and sealed them

165   with nail polish to prevent evaporation.

166   We used a Reichert Polyvar light microscope with 100 to 1000-fold magnification and an affixed

167   Nikon D70 digital camera. We analysed all faecal sample separately, selecting 25 random fields of

168   view (fov) with 400-fold magnification in each of the two subsamples per slide. We counted all spores

169   visible in the fov, allocating them to numbered spore types. We produced a detailed description of

170   each spore type at 1000-fold magnification according to Castellano et al. (1989). The identification of

171   fungal spores was based on Montecchi and Sarasini (2000) and Castellano et al. (1989), and

172   compared to DNA based identification (Urban et al. in prep.).

173   Calculation and Statistics

174   Calculations were based on spore numbers and frequencies. As correction for unequal spore sizes we

175   used volume units (based on average spore dimensions and a spheroid model of spore shape). But as

176   this did not alter most of the results, we returned to the direct and more transparent measure of spore

177   numbers and gave information on spore volume units where informative.

178   Fluctuations in small mammal capture frequencies resulted in highly varying sample sizes regarding

179   small mammal species, trapping sessions, and forest areas. We excluded the two rarest small mammal

180   species ($N = 2$) and the rarest fungal spore types observed (spore count < 50, never more than 2 spores

181   per fov) from all calculations. We used the Kruskal-Wallis Test followed by post hoc Mann-Whitney

182   U-Tests for pair wise comparison and negative binomial regression to account for overdispersion

183   ($VMR = \sigma^2\mu^{-1} >> 1$), non-normality and heterogeneity of variance in our data and analysed the four

184   main and the four less frequent small mammal species separately to reduce the effects of variation in

185   sample size. Variation in spore numbers of individual spore types was high and it was not possible to

186   obtain a normally distributed dataset of individual spore types by transformation.

187 We used the number of spore types as measure of fungal species richness in small mammal food. By

188 transformation (square root) we achieved a normal data distribution and therefore applied parametric

189 tests (MANOVA, TUKEY multiple comparison of means) to evaluate differences between small

190 mammal species, capture session and forest type regarding species richness.

191 To compare the similarity of the distribution of fungal taxa found in samples from *M. glareolus* from

192 all four forest areas we used a hierarchical cluster analysis (dissimilarity calculation with Bray-Curtis-

193 Index and hierarchical clustering with nearest neighbour method using R package "vegan" (Oksanen et

194 al. 2011).

195 For all statistical analyses, we used the open statistical package R (R Development Core Team 2011).

196 To calculate the degree of nestedness of the investigated mutualistic network we used the "nestedness"

197 implementation in the R package "vegan" (Oksanen et al. 2011), which is a direct port of the

198 binmatnest program (Rodríguez-Gironés and Santamaría, 2006). In analogy to thermodynamics, the

199 degree of nestedness as opposed to the entropy of a system is calculated as nestedness temperature.

200 The lower the nestedness temperature, the more nested is the system. Comparison of the nestedness

201 temperature calculated from empirical data and the temperature of three different randomly arranged

202 null models is then used for estimation of the degree of nestedness of a given network. For visual

203 representation of the interrelationships and nested arrangement, we generated a heat map, where the

204 interactions between small mammal species and ECM fungi are represented as the product of the

205 percentage of positive samples and the median spore number of positive samples. This value and the

206 corresponding shade of grey provide an estimation of feeding intensity.

207 **Results**

208 Small mammal species and ECM fungal spore types

209 During the live trapping sessions in the six plots representing three forest types of the Dürrenstein

210 Wilderness Area we captured a total of 400 individuals of eight small mammal species. We achieved a

211 trapping success (100 trap nights[-1] without recaptures) of 2.82 *Apodemus flavicollis*, 2.11 *Myodes*

212 *glareolus*, 1.31 *Sorex araneus*, 1.71 *S. minutus*, 0.18 *Glis glis*, 0.18 *Microtus agrestis*, 0.20 *S. alpinus*,

213 and 0.04 *Pitymys subterraneus* (Fig. 1). The capture results show a typical Central European mountain

214   forest small mammal community with four species comprising about 90 % of the captures and another

215   four species captured in substantially lower numbers. The main four species are: (1) *A. flavicollis*, a

216   forest dwelling murid species with a broad ecological niche; (2) *My. glareolus*, an arvicolid species

217   found in a wide range of forested habitats but favouring moist forests with dense understory and high

218   amounts of coarse woody debris (Corbet et al. 1982); (3) *S. araneus* and (4) *S. minutus*, two frequent

219   Eurasian forest soricids with a broad ecological niche (Mitchell-Jones et al. 1999). Less frequently

220   occurring in the Dürrenstein Wilderness Area are *S. alpinus*, *G. glis*, and two other arvicolid species

221   (*Mi. agrestis and P. subterraneus*) captured on the MF1 and DA plots.

222   In the Rosalia Demonstration Forest we captured 453 individuals of seven species (Fig. 1). Trapping

223   success (100 trap nights^{-1}) was: 6.50 *A. flavicollis*, 6.96 *My. glareolus*, 1.71 *S. araneus*, 1.67 *S.*

224   *minutus*, 1.04 *A. sylvaticus*, 0.29 *Mi. agrestis*, and 0.08 *Micromys minutus*.

225   The small mammal communities consist of the same four main species in both investigation areas, but

226   differ in the accompanying species.

227   We analysed a total of 400 faecal samples (Fig. 1) from the three Dürrenstein Wilderness Area forest

228   areas and 122 samples from the MF2 plots (selection based on sample number per species and

229   session).

230   The sampling design allowed to compare different forest areas as well as different sessions on a

231   species specific level for common small mammal species. To study the feeding ecology of less

232   frequently captured small mammal species, prolonged sampling sessions or inclusion of recaptured

233   animals in the sampling process would be necessary to obtain sufficient sample sizes.

234   During microscopic analysis of the faecal samples we identified 73 distinct spore types. After

235   exclusion of twenty spore types (too rare), we confirmed 20 of the remaining 53 fungal spore types to

236   represent distinct taxa of ECM fungi. Spores from the remaining 33 distinct spore types could not be

237   assigned to any ectomycorrhizal fungal genus and amounted to 12 % of the total number of counted

238   spores. They were excluded from further calculations.

239   The 20 ECM fungal spore types were present in varying amounts in the faecal samples (Fig. 1) and

240   belonged to different phylogenetic groups (Table 1). All recorded ECM fungal taxa produce fleshy

241   fruit bodies and thus to constitute a potential food source for small mammals. We found fourteen taxa

242   of hypogeous fungi, two epigeous taxa, and four which could not be assigned to either type of fruiting

243   with certainty.

244   Ten of the 20 taxa comprised about 97 % of all observed ECM spores (Table 1 ESM), and among

245   these ten three types representing hypogeous basidiomycetes (*Melanogaster broomeianus*,

246   *Hysterangium nephriticum*, and *Hymenogaster sp*.1-5) dominated with more than 15 % of counted

247   spores each. The ratio of spore abundance of ascomycete genera (*Balsamia, Genea, Elaphomyces,*

248   *Tuber*) basidiomycetes was 1:44, whereas the abundance ratio of hypogeous taxa and epigeous taxa

249   was 15:1 (unclassified spore types excluded). The ratio of spore volume units for ascomycetes vs.

250   basidiomycetes was 1:2 and for hypogeous vs. epigeous taxa it was 37:1.

251   Degree of mycophagy of small mammals

252   All investigated small mammal species had ingested ECM fungal spores (Table 2, Fig. 2), but we

253   found considerable variation in median spore numbers between the four main small mammal species

254   (Kruskal-Wallis chi-squared = 107.3, df = 3, p < 0.001). Post hoc pair-wise comparison showed *M.*

255   *glareolus* to defecate significantly higher numbers of ECM spores than the other three main species. *A.*

256   *flavicollis* samples contained significantly more ECM fungal spores than samples from *S. araneus* and

257   *S. minutus* (Table 2). Comparison of the samples from the four rarer small mammal species showed

258   insignificant differences (Kruskal-Wallis chi squared = 2.94, df = 3, p = 0.401; Table 2, Fig. 2).

259   Effect of session, year, and forest area on total spore numbers for single small mammals species

260   Negative binomial regression indicated differing patterns of factor influence for each of the four main

261   small mammal species. The regression model (Total ECM fungal spore no ~ Plot + Session + Year)

262   demonstrated a significant influence of "Plot" (forest type) on the degree of mycophagy of the two

263   *Sorex* species: for *S. araneus*: both "Plot" factor levels MF1 and PF and for *S. minutus* factor level

264   MF1 had a significantly positive effect. Observed effects of "Plot" on *A. flavicollis* and *My. glareolus*

265   were not significant. Factor influence of both "Session" and "Year" was significant for both rodent

266   species, but not for *Sorex* spp. (Table 3). Due to the small sample size of the four rare species and their

267   confinement to single forest areas no statistically reliable comparison was possible.

268   Fungal spore composition in samples from *My. glareolus* indicate site specific ECM communities as

269   illustrated by a hierarchical cluster analysis with samples from all four forest areas (Fig. 3). The

270   dendrogram shows the limited similarity of the MF2 forest area with the Dürrenstein Wilderness Area

271   plots, paralleling the significantly reduced degree and diversity of mycophagy at MF2 (Table 4b).

272   Effect of small mammal species, session, year, and forest area for single ECM fungal spore types

273   In *My. glareolus* samples we found all 20 ECM fungal taxa and in samples of *A. flavicollis* we

274   detected 19 of 20 taxa. Samples from the shrew species contained 16 (*S. araneus*) or 17 (*S. minutus*)

275   ECM fungal taxa. In the samples of the rare small mammal species we determined eight (*A. sylvaticus*,

276   *G. glis*, *S. alpinus*) and ten (*Mi. agrestis*) ECM fungal spore types, respectively.

277   We observed that 11 % of all samples lacked ECM fungal spores, 75 % of all samples contained one

278   to four taxa, and the remaining 14 % contained five to nine taxa of ECM fungi. MANOVA and

279   TUKEY post hoc test with following model: No of ECM spore types ~ Small mammal species *

280   Forest Area * Session (Table 4a) revealed all three variables to have significant influence on the

281   dependent variable. Samples from *M. glareolus* showed the highest number of spore types ($p < 0.001$),

282   but we did not detect significant differences between the other three common small mammal species.

283   For the variable "Plot", we found samples from the Rosalia Demonstration Forest (MF2) to have

284   significantly lower numbers of spore types, while the three forest areas within the Dürrenstein

285   Wilderness Area did not exhibit significant differences. For the variable "Session" we found no

286   significant differences between summer and autumn samples (Table 4b).

287   We explored the influence of environmental factors on ECM fungal spore types (Table 1, ESM Table

288   1 and 2) by negative binomial regression with the factors "Small mammals species", "Plot", and

289   "Session" for every spore type separately (Table 5). We restricted this evaluation to data from

290   Dürrenstein Wilderness Area to reduce unaccounted variation resulting from geographical separation

291   and different habitat characteristics. For *Cortinarius sp*. the given set of coefficients was invalid, so

292   we omitted the "Session" factor (in Table 5 denoted as not calculated), to reduce the number of

293   factors.

294   The factor "Small mammal species" significantly explained variation in observed spore numbers in 15

295   out of 20 spore types, but each spore type differed in results regarding the single factor levels.

296   According to the z-values, *My. glareolus* samples contained significantly more spores of twelve spore

297   types than any other of the three common small mammal species (Table 5). Two ECM fungal spore

298   types, *Tuber* aff. *puberulum* and *Russulaceae* 2, were significantly more abundant in samples of *S.*

299   *minutus* and *S. araneus,* , respectively than in samples from the rodent species while six types (*E.*

300   *asperulus*, *Chamonixia caespitosa*, *Melanogaster broomeianus*, *Hysterangium nephriticum*,

301   *Hymenogaster sp.*1-5, *Octaviania asterosperma*) had significantly lower z-values in *Sorex* samples

302   (Table 5). For the regression factor "Plot" we found that MF and PF had a similar effect on the number

303   of observed fungal spores for ten ECM fungal spore types. In six ECM fungi (*Balsamia sp.*,

304   *Elaphomyces asperulus*, *E*. cf. *muricatus*, *Russulaceae* 2, *C. caespitosa*, *Hymenogaster sp.*1-5)

305   calculated z-values were lower in samples from MF and PF than in samples from DA while for *T.*

306   *rufum*, *Russulaceae* 1, *Boletaceae*, *M. broomeianus* , and *H* cf. *hessei* z-values were higher for

307   samples from MF and PF (Table 5). For most of these spore types, results were significant for both

308   MF and PF, but *Russula* sp.1 and *H.* cf. *hessei* were most abundant in samples from PF and

309   *Hymenogaster sp.* 1-5 was least abundant in samples from this plot. *T. rufum*, *Boletaceae*, and *M.*

310   *broomeianus* were most abundant in samples from MF, while *Balsamia sp.* and *E.* cf. *muricatus* were

311   least abundant in MF samples. For the remaining ten ECM fungal spore types the factor "Plot" did not

312   explain variation in observed numbers of ECM spores.

313   The factor "Session" explained spore number variation in five ECM fungi, for all of them we found

314   more spores in Autumn samples. Regarding the sampling years we found significantly more spores of

315   *E.* cf. *muricatus*., *Russulaceae* 1, *Boletaceae*, *M. broomeianus*, *Cortinarius sp.*,

316   *Hymenogaster/Cortinarius sp.*, *H.* cf. *hessei* in samples from 2006, while observed spore numbers of

317   *Balsamia sp.*, *T.* aff. *puberulum*, *E. asperulus*, *Russulaceae* 2, *O. asterosperma*, and *Hymenogaster*

318   *sp.*1-5 were significantly higher in 2007 (Table 5).

319   Food web nestedness

320   The heat map (Fig. 4) showed an arrangement typically found in nested communities. In our case a

321   subset of fungi was consumed more frequently (indicated by higher values and darker shade) and by

322   more small mammal species while other ECM fungi seemed to be taken up in low numbers but

323   widespread throughout the small mammal community and finally there were five ECM fungi

324   (*Pezizales* sp., *Tuber rufum*, *Elaphomyces granulatus*, *Hymenogaster/Cortinarius sp.*, *Hymenogaster*

325   cf. *hessei*) which were restricted to only a few small mammal species.

326   The calculated system temperature was $T = 3.76$. The calculated mean temperatures of three null

327   models were $T_1 = 32.60$; $T_2 = 30.92$; $T_3 = 22.32$. The statistic comparison resulted in rejection ($p <$

328   0.001 for all three null models) of the null hypothesis (H_0 = Matrix of food web randomly created) and

329   supported hypothesis (d).

330   **Discussion**

331   Varying degree of mycophagy in the small mammal community

332   The presence of fungal spores of ECM fungi in samples of all eight small mammal species studied

333   confirm and extend earlier reports on mycophagy in the vole *My. glareolus*, the murid *A. flavicollis*

334   (Blaschke and Bäumler 1989) and, as shown very recently, in the insectivores *S. araneus* and *S.*

335   *minutus* (Kataržytė and Kutorga 2011). The data about mycophagy in *G. glis* and *S. alpinus* are the

336   first of their kind, according to the best of our knowledge.

337   Significant differences in numbers of fungal spores ingested by different small mammal species

338   indicate a species-specific degree of mycophagy in the small mammal community of Central European

339   mountain forests, confirming hypothesis (a). Furthermore, mycophagy of at least four of the

340   investigated small mammal species varies seasonally and/or between forest areas, so hypotheses (b)

341   and (c) are supported as well.

342   Our observations on the distribution of spores of hypogeous fungi in the faeces of all eight small

343   mammal species parallel results from other continents: There is one species (*My. glareolus*)

344   consuming a considerable variety and amount of ectomycorrhizal fungi across all forest types and

345   trapping sessions and there are many others which use this food source periodically or with a narrower

346   range of consumed species (Fig. 4). Vernes and Dunn (2009) found the same pattern across a

347   landscape gradient in eastern Australia, where the bush rat as the main mycophagous species was

348   accompanied by many other species with a lesser degree of mycophagy.

349   *My. glareolus* exhibits the highest degree of mycophagy in terms of quantity and species richness, but

350   its feeding habits appear to be less specialised on fungi compared to its North-American cousins *My.*

351   *californicus* and *My. gapperi*, which are considered obligate mycophagists (Ure and Maser 1982). The

352   number of spores observed in the *My. glareolus* samples varies between sessions, forest types and

353   individual samples, and there are other food items present in varying amounts throughout the year.

354   The important seasonal variation in the quantity and diversity of ECM fungal species consumed by

355   *My. glareolus* suggests that supply of this food source is too unpredictable in the habitats investigated

356   to allow high specialisation. However, given the overall abundance and diversity of fungal spores

357   ingested, *My. glareolus* might be regarded as preferentially mycophagous (Claridge and Trappe 2005).

358   For *A. flavicollis* fungi seem to be an important food source in times of abundance, but with only one

359   tenth of ECM spores observed compared to *My. glareolus* the species has to be regarded as casually or

360   opportunistically mycophagous (Maser et al. 2008). Given the less burrowing lifestyle of *A. flavicollis*

361   this is not surprising, however, some animals defecated substantial amounts of fungal material (up to

362   5000 spores per 50 fov). We can therefore conclude that fungi are consumed in considerable

363   quantities, when available, but *A. flavicollis* does not rely on them as a food source.

364   We found significantly less ECM spores in the faeces of all three shrew species than in samples from

365   the two main rodent species, but there are some samples with very high numbers of ECM fungal

366   spores. While the number and aggregation of ECM spores in the rodent samples indicate mycophagy,

367   it remains unclear whether the shrews forage actively for fungi. Their insectivorous feeding habit

368   might cause them to ingest fungal spores when they prey on mycophagous invertebrates (Fogel and

369   Trappe 1978). Indirect spore uptake has been shown for other insectivorous species, e. g. *Antechinus*

370   *spp.* (Vernes 2007) and is considered as accidental mycophagy (Claridge and Trappe 2005). But, the

371   high spore numbers we observed in some samples rather indicate direct consumption of fungal fruit

372   bodies (especially *Tuber* aff. *puberulum*, *Russulaceae* 2), albeit possibly infested with insect larvae.

373   Therefore *Sorex* spp might better be considered selective occasional mycophagists. Efficiency of spore

374   dispersal can be assumed to depend on a variety of behavioural properties, such as microhabitat

375   preferences and home range size. Even as secondary consumers, shrews can act as spore vectors by

376   distributing the viable spores over a larger distance than the primary invertebrate consumers might do.

377   Thus, shrews might play an underestimated role in spreading fungal spores.

378   *A. sylvaticus*, *G. glis*, and *Mi. agrestis* can be regarded as opportunistically mycophagous. Spore

379   numbers found in the scats of *A. sylvaticus* are within the same range as found for *A. flavicollis*. With

380   regard to mycophagy, no niche differentiation could be detected between the two *Apodemus* species.

381   *G. glis* seems to descend to the forest floor to dig for truffles despite its mainly arboreal lifestyle, like

382   the opportunistically mycophagous Brushtail possums (*Trichosurus vulpecula*) of Southeastern

383   Australia (Claridge and Lindenmayer 1998) or various squirrel species (Bertolino et al. 2004; Vernes

384   et al. 2004). Due to its rather large home range (up to 7 ha for males; Ściński and Borowski 2008), *G.*

385   *glis* might be an important long distance vector.

386   Regarding the ecological niche of *Mi. agrestis* and *P. subterraneus* (strictly ground-dwelling vole

387   species with a life style comparable to *My. glareolus;* (Mitchell-Jones et al. 1999) we expected higher

388   amounts of ECM spores in their samples. From the overall results it is clear that their low degree of

389   mycophagy compared to *My. glareolus* can not be explained by a shortage of ECM fungi during the

390   sampling sessions, but more samples are needed to draw conclusions. We suggest sampling in areas of

391   known abundance of these species to achieve representative sample sizes.

392   Diversity of ECM fungi consumed

393   The majority of samples contained between one and four ECM fungal spore types, indicating that

394   small mammals consume a variety of ECM fungi if available. This polyphagous feeding habit reduces

395   dependence on differing fruiting times of the ECM species, making the fungal food source more

396   reliable. Other potential advantages might be a more balanced nutrition as well as reduced searching

397   effort.

398   Only two of the hypogeous fungal taxa detected during this study (*Hysterangium nephriticum* and

399   *Elaphomyces muricatus*) had been discovered during previous surveys (Kovacs 2001) in the

400   Dürrenstein Wilderness Area. The study of Kovacs (2001) revealed a high diversity of epigeous ECM

401   fungi in the primeval forest area, but the fact that we found a larger proportion of spores of hypogeous

402 fungi shows that small mammals prefer those, even if mushrooms are abundant. The reason for this

403 might be the more stable supply of truffles or the reduction of toxicity in hypogeous fungi (Claridge

404 and Trappe 2005).

405 The relatively low frequency of spores of hypogeous ascomycetes of the genus *Elaphomyces* in the

406 faecal samples is in contrast to fortuitous ascocarp records, but can be explained by the specific

407 dispersal strategy of this genus: Unlike other hypogeous fungi, the spores form a powdery mass, which

408 is exposed to the wind during manipulation by mycophagists, that typically feed on the peridium

409 (Maser et al. 2008).

410 Forest succession and diversity of small mammal and hypogeous fungal communities

411 The four investigated forest types differ regarding both the small mammal and the fungal components

412 of the mycophagy network. The observed variations in abundance, species richness and composition

413 of egested spores of hypogeous fungi indicate site specific ECM communities to which the

414 mycophagists can adopt.

415 The feeding habit of *My. glareolus*, the key mycophagist, does not differ significantly between the

416 primeval forest and the sustainably managed forest plots in the Dürrenstein Wilderness Area,

417 indicating that forest management practices can be compatible with the persistence of mycophagous

418 relationships. On the succession plot (DA) mycophagy by *My. glareolus* is slightly lower in terms of

419 spore abundance and diversity, but these differences are statistically not significant. The structurally

420 diverse succession plots, with a tessellate pattern of graminoid and herbal vegetation and patches of

421 forest regeneration, is likely to provide a diversity of alternative food sources, potentially reducing the

422 fidelity of the mycophagists to their fungal food source, albeit without challenging the persistence of

423 the mycophagy network. Six spore types of hypogeous fungi were observed most abundant in samples

424 from the DA, while several others were found less abundant than in more mature forests (Table 5).

425 The differences in community composition between the DA and the MF1 and PF (Fig. 3) suggest that

426 a specific community of hypogeous fungi is associated with the successional stage of the DA.

427 Reduced mycophagy in the Rosalia Demonstration forest plots (MF2) may in part be due to the

428 composition of the dense ground vegetation of mostly arbuscular mycorrhizal host plants (grasses,

429   ferns, bramble). An abundant ground vegetation appears to provide alternative food sources for *My.*

430   *glareolus*, sustaining high population densities but reducing its dependency on fungi, and can compete

431   with trees and their ectomycorrhizal associates for water and nutrients (e. g. Dodet et al. 2011)

432   potentially reducing the resources allocated to sporocarp production. This leads us to the hypothesis,

433   that more or less mycophagous feeding habits may be part of feedback loops which result in either

434   rapid regeneration of predominantly ECM forest or long-term persistence of grassy and shrubby

435   vegetation.

436   Several additional factors may account for differences in intensity of mycophagy and in diversity of

437   hypogeous fungi consumed observed between the two study regions. The managed forest plots in both

438   sites are comparable with regard to tree species composition, tree age and practices of forest

439   management, but in the Rosalia Demonstration Forest they are surrounded by intensively managed

440   forests in a densely populated area. The fungal species richness of the investigated managed forest in

441   the Dürrenstein Wilderness Area (MF1) shows the positive impact of large extensions of sustainably

442   managed forests and the close proximity of primeval forest, where mycophagists potentially vector

443   ECM fungal spores between managed and unmanaged forest. Furthermore, the humid, suboceanic

444   climate is likely to promote ECM fungal diversity and continuous productivity in the Dürrenstein

445   Wilderness Area, while fungal sporocarp production is limited by periods of draught characteristic for

446   the more continental climate of the Rosalia Demonstration Forest.

447   Mutualistic network structure

448   The limitations of the taxonomic resolution achievable by light microscopy and uncertainties

449   connected to rarer spore types may result in an oversimplified representation of the actual fungi-

450   mycophagist network, but general patterns are clearly visible: The high degree of nestedness suggests

451   that the trophic relationships involving generalists and specialists are arranged in a non-random way.

452   i.e. that they are highly organised. This result indicates that the analogies of mycophagy with other

453   resource – service mutualistic networks such as pollination or frugivory result in very similar

454   structural characteristics, and that conclusions reached for more easily accessible networks (e. g. plant-

455   pollinator or plant-frugivore; Bascompte et al. (2003) might also apply to the fungi-mycophagist

456   relationship. Nestedness can provide feedback loops and promote diversity by increasing the number

457   of coexisting species (Bastolla et al. 2009). Mycophagy is considered to enhance functionality and

458   resilience of forests (Johnson 1996; Claridge 2002), and nestedness appears to contribute to these

459   ecosystem functions.

460   Conclusions and Management considerations

461   The animal and fungal species involved in mycophagy networks differ among continents, but some

462   fundamental characteristics appear to be surprisingly similar. Phylogenetically diverse communities of

463   animal species (Johnson 1996) disperse a highly diverse assemblage of fungal species, among which

464   the typically ECM hypogeous fungi are predominant. Varying levels of specialisation and dependency

465   are found on the higher trophic level, while hypogeous fungi fully rely on animal vectors for dispersal.

466   In the investigated Central European forest ecosystems no obligate mycophagist is present, but all

467   small mammal species studied are mycophagous to some extent. The most active mycophagists are not

468   endangered, unlike Australian mycophagous species (e. g. Johnson 1996; Green et al. 1999; Vernes

469   2003). The dietary ecology of small mammals including *Sorex* spp. and *G. glis* appears to be more

470   versatile than previously reported. The exclusive view of rodents as predators of tree seeds and

471   seedlings needs to be revised.

472   Hypogeous ECM fungi seem to be present in considerable diversity and abundance. At least 14

473   certainly hypogeous fungal taxa depend on dispersal by mycophagists in the investigated areas, 12 of

474   them reported for the first time. Furthermore, the unexpected high frequency of *Chamonixia*

475   *caespitosa* spores shows, that mycophagy studies can provide new data about the ecology and

476   distribution of hypogeous fungi, one of the least known groups of macrofungi. More data about these

477   partly red-listed species are needed not at least for a better assessment of their conservation status.

478   In contrast to mycorrhizal symbiosis, mycophagy is not yet widely acknowledged as a process

479   contributing to forest vitality, productivity and resilience. This investigation demonstrates that a non-

480   negligible part of the ectomycorrhizal communities of managed and unmanaged forests in different

481   successional stages relies on animal dispersal. A specific community of ectomycorrhizal hypogeous

482   fungi appears to be associated with younger trees in forest regeneration sites after large scale

483   disturbance. We hypothesise that 1) the availability of animal vectored ectomycorrhizal inoculum is

484   important for forest regeneration particularly in situations approximating primary succession and 2)

485   that mycophagy interacts with successional trajectories leading either to rapid development of

486   predominantly ECM forest or long term persistence of predominantly arbuscular mycorrhizal ground

487   vegetation.

488   Impact of timber harvest on fungi and mammal diets was studied extensively in the Northwestern

489   United States (Carey and Harrington 2001; Carey et al. 2002; Luoma et al. 2004; Gitzen et al. 2007).

490   As we found similar relationships between small mammals and fungi in Central Europe, we can adopt

491   the recommendations inferred: A sustainable management retaining mature trees and protecting the

492   forest soil limits disturbance to a level that does not impair the persistence of mycophagy networks,

493   leaving intact sources of mycorrhizal fungi and alternative food for small forest mammals (Dell 2002;

494   Wiensczyk et al. 2002). Additionally, Wiensczyk et al. (2002) strongly recommend the retention of

495   coarse woody debris, since Amaranthus et al. (1994) found that fruiting of hypogeous fungi was linked

496   to the presence of coarse woody debris. Maintaining a natural composition of both small mammal and

497   hypogeous fungal communities will promote forest health and contribute to forest resilience (Jacobs

498   and Luoma 2008).

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626

627   **Fig. 1** Small mammal community present in the investigated forest plots (left, DWA – Dürrenstein

628   Wilderness Area, RDF – Rosalia Demonstration Forest) and ECM fungi present in small mammal

629   faecal samples (right), *Af - Apodemus flavicollis, Mg - Myodes glareolus, Sar - Sorex araneus, Sm - S.*

630   *minutus, Ma - Microtus agrestis, Ps - Pitymys subterraneus, Sal - S. alpinus, As - A. sylvaticus, Mm -*

631   *Micromys minutus*, abbreviations of fungal taxa given in table 1

632   **Table 1** Systematic classification and ecology of ECM fungal groups determined. Informations about

633   ecology based on Montecchi and Sarasini (2000).

634   **Table 2** Number of samples (N), median number of ECM spores observed per 50 fov, and pair-wise

635   comparison of observed ECM spore numbers with Kruskal-Wallis post hoc procedure for eight small

636   mammal species, significant differences indicated in **bold**

637   **Fig. 2** Boxplot of total ECM spore numbers (log scaled, zeros omitted) for small mammal species

638   from DWA (small mammal species abbreviations as in Fig. 1), whisker length = 1.5 SD, outliers

639   depicted as open circles

640   **Table 3** Coefficients of negative binomial regression model for total numbers of ECM spores for the

641   four main small mammal species

642   **Fig. 3** Hierarchical cluster analysis based on ECM fungal spores in *My. glareolus* samples from all

643   four forest areas, clustering method – nearest neighbour, height – amount of dissimilarity, maximum =

644   1, default rooting)

645   **Table 4** Differences in number of defecated ECM spore types between the four main small mammal

646   species, forest areas and sessions

647   (a) Results of MANOVA

648   (b) Results of TUKEY multiple comparison of means

649   **Table 5** Coefficients of negative binomial regression for single ECM types (Z-values calculated with

650   negative binomial regression), data from Dürrenstein Wilderness Area only

651   **Fig. 4** Heat map of ECM fungal spore egestion by small mammal species illustrating the nested

652   arrangement of the mycophagist – ECM fungus network (values = % of positive samples * median of

653   spore counts in positive samples; for abbreviations of fungal taxa see Table 1)
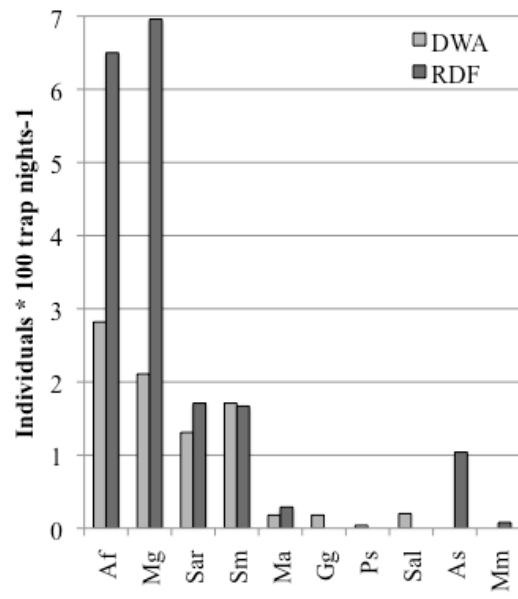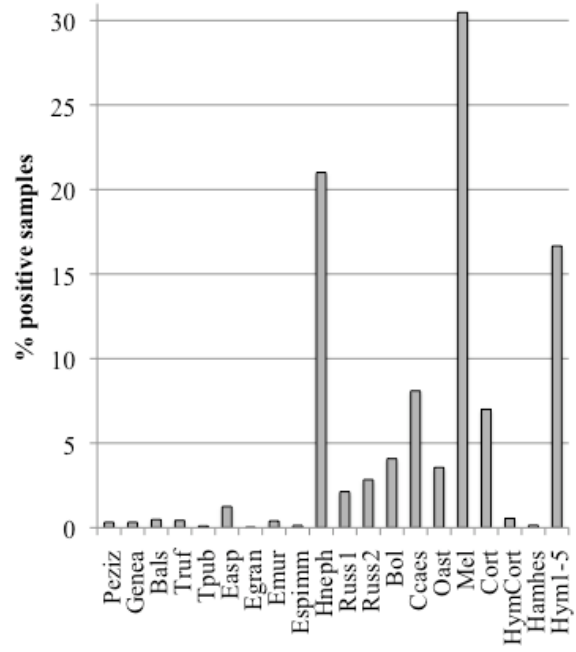
|	Order	Family	Type	Abbre-viation	Fruiting habit	Ecology	Found as Sporocarp
Asco-mycota	*Pezizales*	*Pyronema-taceae?*	*Pezizales sp.*	Peziz	epigeous?		
		Pyronema-taceae	*Genea sp.*	Genea	hypogeous		
		Helvellaceae	*Balsamia sp.*	Bals	hypogeous		
		Tuberaceae	*Tuber rufum agg.*	Truf	hypogeous	"widespread in all environments, associated with both to coniferous and deciduous trees, from spring to late autumn"	
			Tuber aff. puberulum	Tpub	hypogeous		
	Eurotiales	*Elaphomy-cetaceae*	*Elaphomyces cf. asperulus*	Easp	hypogeous	"not very common, preferably in coniferous or mixed woods, under *P. abies* or *A. alba*, more rarely in deciduous woods, growing all the year"	
			Elaphomyces granulatus	Egran	hypogeous	"common in coniferous woods, in autumn-winter, generally rather deep in the soil, unter the needle sheet, up to 1200 m altitude"	x
			Elaphomyces cf. muricatus	Emur	hypogeous	"rather common, in deciduous or coniferous woods, generally rather deep in the humus, mainly in summer and autumn, up to 1200 m altitude"	
			Elaphomyces sp. imm.	Espimm	hypogeous		
Basidio-mycota	*Hysteran-giales*	*Hysterangia-ceae*	*Hysterangium nepriticum*	Hneph	hypogeous	"generally under deciduous trees, especially in woods with *F. sylvatica*, autunmn"	x
	Russulales	*Russula-ceae*	*Russulaceae 1*	Russ1	?		
			Russulaceae 2	Russ2	?		
	Boletales	*Boletaceae*	*Chamonixia caespitosa*	Ccaes	hypogeous	"quite rare species, found from July to October in mountain coniferous forests (*Picea, Abies*)"	
		Melano-gastraceae	*Octaviania asterosperma*	Oast	hypogeous	"very frequent species, sometimes nearly semi-epigeous, in deciduous or mixed woods, at various altitudes and climates"	x
			Boletaceae sp. (B. edulis?)	Bol	epigeous?		
			Melanogaster broomeianus	Mel	hypogeous	"very common species in all kind of fresh and shadowy woods, from the plain to the mountains, from spring to late autumn, …mainly semi-epigeous"	
	Agaricales	*Cortinaria-ceae/ Hymeno-gastraceae*	*Cortinarius sp.*	Cort	epigeous		
			Hymenogaster sp./ Cortinarius sp.	HymCort	epigeous?		
			Hymenogaster cf. hessei	Hymhes	hypogeous		
			Hymenogaster sp.1-5	Hym1-5	hypogeous		

Species	N	Total median no of ECM spores/50 fov	Pair wise comparison with	W	P-value
My. glareolus	167	79	*A. flavicollis*	18789.5	**< 0.001**
			S. araneus	8930.5	**< 0.001**
			S. minutus	12319.0	**< 0.001**
A. flavicollis	153	7	*My. glareolus*	7763.5	**< 0.001**
			S. araneus	6516.0	**0.004**
			S. minutus	9236.0	**< 0.001**
S. araneus	66	4	*A. flavicollis*	3978.0	**0.004**
			My. glareolus	2091.5	**< 0.001**
			S. minutus	3171.5	0.395
S. minutus	89	4	*A. flavicollis*	4915.0	**< 0.001**
			My. glareolus	2544.0	**< 0.001**
			S. araneus	2702.5	0.395
A. sylvaticus	8	4.5	*G. glis*	40.5	0.084
			Mi. agrestis	91.5	0.752
			S. alpinus	91.5	0.962
G. glis	8	11	*A. sylvaticus*	103.5	0.084
			Mi. agrestis	57.5	0.281
			S. alpinus	55.0	0.196
Mi. agrestis	11	8	*A. sylvaticus*	106.5	0.751
			G. glis	30.5	0.281
			S. alpinus	51.5	0.813
S. alpinus	11	5	*A. sylvaticus*	88.5	0.962
			G. glis	25.0	0.196
			Mi. agrestis	58.5	0.813
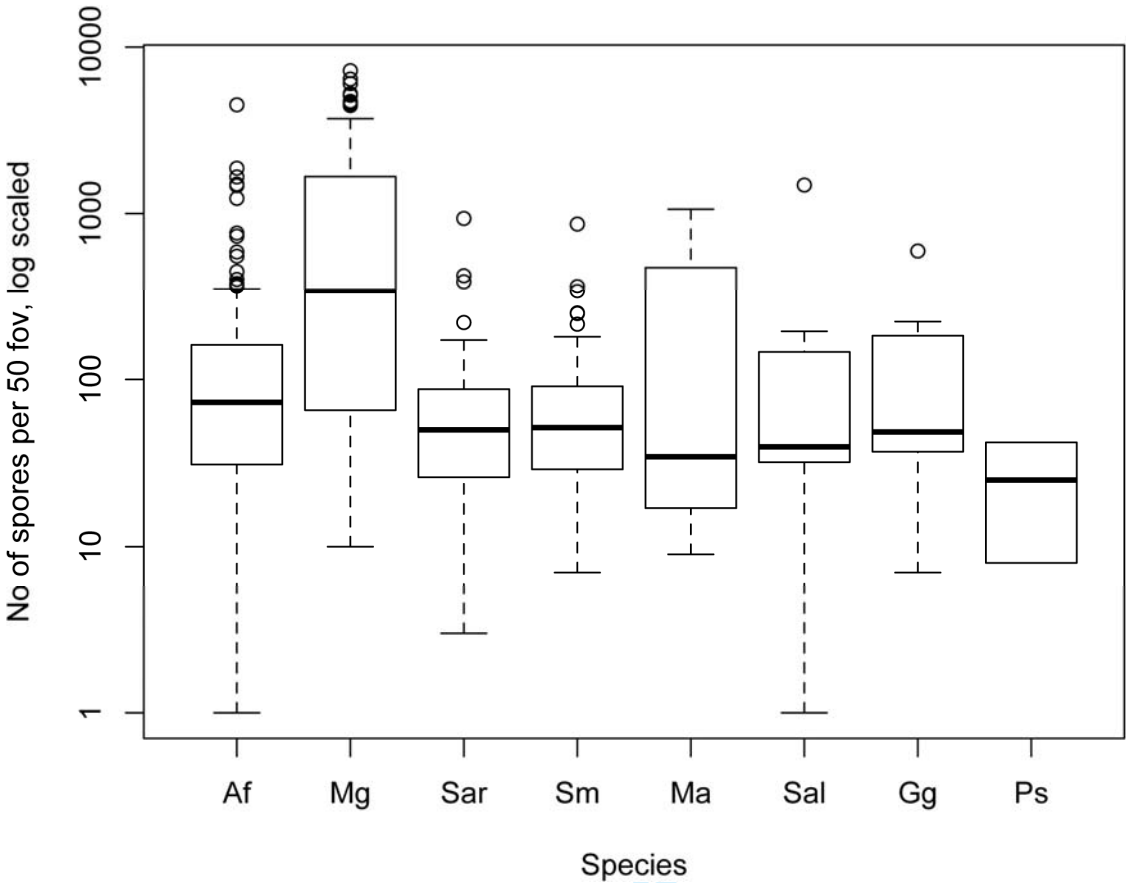
Factor	*A. flavicollis*	*My. glareolus*	*S. araneus*	*S. minutus*
Intercept	**-4.036*** **	**-3.313*** **	0.518	1.876'
Plot				
DA	0	0	0	0
MF2	-1.061	-1.036	-0.225	0.195
MF1	0.644	0.973	**4.576*** **	**2.418***
PF	1.054	0.507	**4.226*** **	0.854
Session				
Autumn	0	0	0	0
Summer	**-4.516*** **	-0.347	-0.718	0.130
Year				
2006	0	0	0	0
2007	**4.044*** **	**3.324*** **	-0.516	-1.874'

Parameters that were significant at P = 0.05 are in bold,
P-values represented as follows:' P = 0.1/* P = 0.05/** P = 0.01/*** P = 0.001
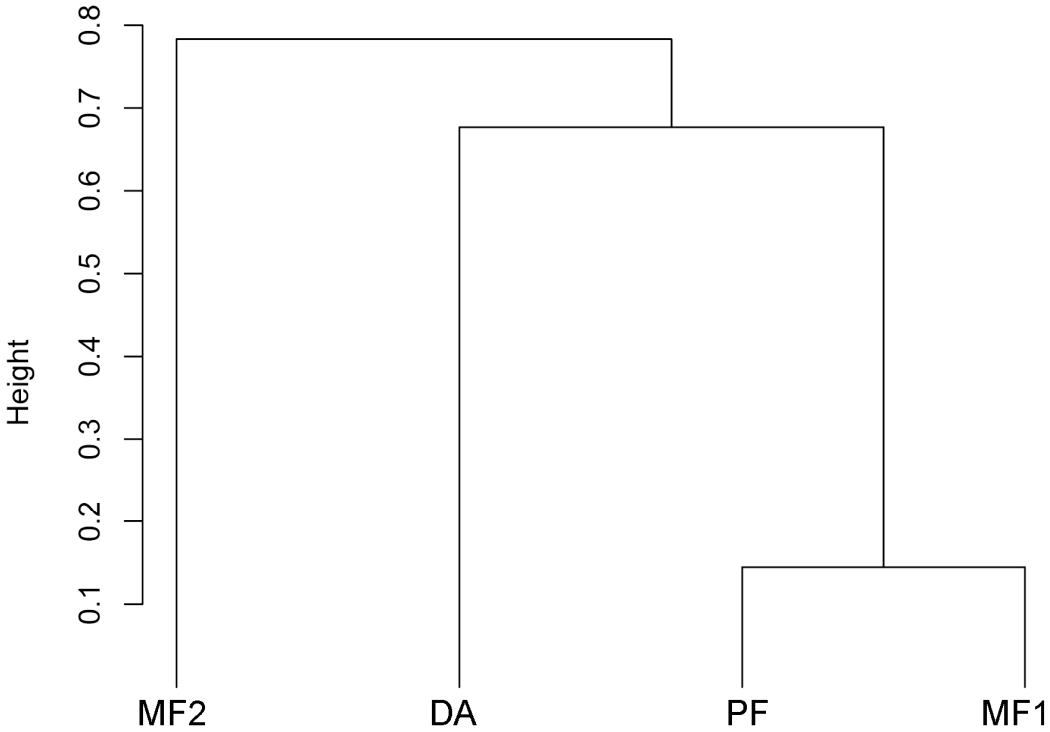
Factor	Df	Sum Sq	Mean Sq	F value	P value
Species	3	33.6	11.2	30.4	**< 0.001***
Plot	3	6.7	2.2	6.1	**< 0.001***
Session	1	1.7	1.7	4.6	**0.036***
Species:Plot	9	6.7	0.7	2.0	**0.037***
Species:Session	3	3.9	1.3	3.6	**0.014***
Plot:Session	3	0.3	0.1	0.3	0.838
Species:Plot:Session	8	2.9	0.4	1.0	0.447
Residuals		450	165.9	0.4	

Parameters that were significant at P = 0.05 are in **bold**,
P-values represented as follows: ' P = 0.1/* P = 0.05/** P = 0.01/*** P = 0.001

	Mean no of ECM fungal spore types	Difference	Lower	Upper	P value
Species					
My. glar. / A. flav.	3.3 / 2.0	0.436	0.263	0.610	**< 0.001***
My. glar. / S. aran.	3.3 / 1.7	0.632	0.860	0.405	**< 0.001***
My. glar. / S. min.	3.3 / 1.5	0.625	0.830	0.419	**< 0.001***
A. flav. / S. aran.	2.0 / 1.7	0.196	0.425	-0.033	0.124
A. flav. / S. min.	2.0 / 1.5	0.188	0.396	-0.019	0.089'
S. aran. / S. min.	1.7 / 1.5	0.008	0.247	0.262	0.999
Plot					
MF2 / DA	1.6 / 2.1	0.243	0.451	0.035	**0.015***
MF1 / DA	3.0 / 2.1	0.084	0.118	0.287	0.705
PF / DA	2.7 / 2.1	0.035	0.235	0.165	0.969
MF1 / MF2	3.0 / 1.6	0.327	0.122	0.532	**< 0.001***
PF / MF2	2.7 / 1.6	0.208	0.006	0.411	**0.041***
PF / MF1	2.7 / 3.0	0.119	0.315	0.077	0.398
Session					
Su / Au	2.2 / 2.3	0.115	0.225	0.005	0.999
Species : Plot (only significant pairs given)					
My. glar. MF1 / My. glar. MF2	4.4 / 1.8	0.609	0.211	1.008	**< 0.001***
My. glar. PF / My. glar. MF2	4.0 / 1.8	0.496	0.078	0.915	**0.005****
Species : Session (only significant pairs given)					
My. glar. Su / My. glar. Au	2.9 / 3.7	0.311	0.599	0.024	**0.023***

Parameters that were significant at P = 0.05 are in bold,
P-values represented as follows: ' P = 0.1/* P = 0.05/** P = 0.01/*** P = 0.001

	Peziz	Genea	Bals	Truf	Tpub	Easp	Egran	Emur	Espimm	Hneph
Intercept	0.000	-1.306	**-2.700****	1.471	-1.848'	**-4.192*****	0.000	**-2.540***	-0.009	-1.447
Species										
A. flavicollis	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
My. glareolus	0.000	**4.257*****	**2.091***	1.551	0.759	**3.452*****	0.000	**4.053*****	-1.048	**5.845*****
S. araneus	0.000	-0.467	-1.410	0.000	0.549	**-2.952****	0.000	0.000	**2.854****	1.900'
S. minutus	0.000	-0.154	0.210	0.000	**3.502*****	**-2.941****	0.000	1.406	1.082	**-2.770****
Plot										
DA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MF1	0.000	0.011	**-2.123***	**2.840****	-1.296	**-2.282***	0.000	**-2.509***	0.008	0.140
PF	0.000	-0.380	-1.098	1.526	-0.129	**-1.982***	0.000	-0.747	0.008	-0.315
Session										
Autumn	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Summer	0.000	-1.299	-1.344	-1.020	**2.415***	-0.705	1.262	-0.584	-1.430	1.446
Year										
2006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2007	0.000	1.304	**2.699****	-1.472	1.846'	**4.192*****	0.000	-1.968'	0.009	1.447

	Russ1	Russ2	Bol	Ccaes	Oast	Mel	Cort	HymCort	Hymhes	Hym1-5
Intercept	**6.754*****	**5.093*****	**2.178***	1.435	**4.316*****	**4.088*****	**2.686****	0.001	**-2.322***	**-3.448*****
Species										
A. flavicollis	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
My. glareolus	**5.092*****	**6.359*****	**4.008*****	**2.231***	0.250	**2.569***	**6.234*****	0.590	-1.072	**3.598*****
S. araneus	0.557	1.309	0.593	**-2.293***	**-3.922*****	**-5.011*****	0.414	1.422	-0.754	**-3.481*****
S. minutus	-1.239	1.009	0.930	**-4.788*****	**-4.888*****	**-5.605*****	0.035	0.916	1.350	**-4.752*****
Plot										
DA	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MF1	0.673	**-4.168*****	**3.138****	**-2.653****	1.252	**3.479*****	1.875'	0.000	1.386	-1.240
PF	**3.350*****	**-3.132****	-1.030	**-3.096****	0.314	1.295	0.764	0.000	**2.268***	**-2.490***
Session										
Autumn	0.000	0.000	0.000	0.000	0.000	0.000	not calc.	0.000	0.000	0.000
Summer	-0.635	**5.311*****	0.395	**2.293***	**5.997*****	**5.155*****		0.000	-0.020	-1.653'
Year										
2006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2007	**-6.755*****	**5.091*****	**-2.180***	-1.432	**3.065*****	**-4.004*****	**-2.687****	**-2.175***	**-3.641*****	**3.452*****

Parameters significant at P = 0.05 are in bold,
P-values represented as follows:' P = 0.1/* P = 0.05/** P = 0.01/*** P = 0.001

	My. glareolus	*A. flavicollis*	*S. minutus*	*S. araneus*	*Mi. agrestis*	*G. glis*	*S. alpinus*	*A. sylvaticus*
Mel	1922	216	95	234	575	1825	382	38
Bals	43	75	43	6	63	75	73	150
Bol	97	22	54	13	25	100	9	25
Hym1-5	793	784	51	140	413	75	109	0
Hneph	2274	30	16	115	3288	56	0	0
Tpub	5	7	18	6	13	75	0	0
Ccaes	484	231	12	102	13	0	245	100
Easp	108	135	7	17	225	0	0	113
Russ2	73	52	61	96	0	25	9	100
Cort	424	38	38	30	38	0	45	0
Oast	80	24	3	51	0	338	0	150
Russ1	175	25	8	262	0	0	18	0
Genea	77	4	5	2	25	0	0	0
Hymhes	13	10	4	2	0	0	0	0
HymCort	24	1	3	515	0	0	0	0
Espimm	2	8	32	21	0	0	0	0
Emur	35	4	12	0	0	0	0	0
Truf	42	12	0	0	0	0	0	0
Egran	7	0	0	0	0	0	0	100
Peziz	132	3	0	0	0	0	0	0

colour code:	0	1 - 9	10 - 99	100 - 999	> 1000

cell value = % positive samples x median of positive samples

Electronic Supplementary Material (ESM) 1

The interrelationship of mycophagous small mammals and ectomycorrhizal fungi in primeval, disturbed and managed Central European mountainous forests

Susanne Schickmann, Alexander Urban, Katharina Kräutler, Ursula Nopp-Mayr, Klaus Hackländer

ESM1: Numbers of spores of different taxa of ECM fungi egested by small mammal species.

min/max = minimum/maximum no of spores per sample; %ps = percent of positive samples; mps = median spore number in positive samples. For abbreviations of fungal taxa see tab. 1.

	A. flavicollis				M. glareolus				S. araneus				S. minutus				% of
N	131				119				47				74				total
	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	ECM
Peziz	0	3	1	3	0	157	1	157	0	0	0	0	0	0	0	0	0.32
Genea	0	2	4	1	0	43	19	4	0	1	2	1	0	3	3	2	0.32
Bals	0	8	25	3	0	17	14	3	0	7	6	1	0	4	22	2	0.48
Truf	0	5	3	4	0	71	17	3	0	0	0	0	0	0	0	0	0.43
Tsp	0	11	7	1	0	5	3	2	0	2	4	2	0	7	18	1	0.10
Easp	0	69	27	5	0	82	27	4	0	3	9	2	0	3	7	1	1.25
Egran	0	0	0	0	0	12	2	4	0	0	0	0	0	0	0	0	0.03
Esp	0	4	1	4	0	142	6	6	0	0	0	0	0	7	3	5	0.40
Espimm	0	4	4	2	0	2	1	2	0	9	4	5	0	32	5	6	0.13
Hyst	0	144	15	2	0	2766	37	62	0	318	19	6	0	5	8	2	21.01
Russ1	0	2	10	3	0	149	22	8	0	86	6	41	0	5	3	3	2.13
Russ2	0	5	26	2	0	292	24	3	0	711	32	3	0	7	20	3	2.84
Bol	0	5	11	2	0	568	24	4	0	31	13	1	0	335	11	5	4.08
Ccaes	0	264	21	11	0	685	40	12	0	43	9	12	0	44	4	3	8.09
Oast	0	399	8	3	0	295	16	5	0	21	4	12	0	1	3	1	3.56
Mel	0	326	48	5	0	1455	63	31	0	387	43	6	0	319	27	4	30.47
Cort	0	7	19	2	0	615	35	12	0	9	15	2	0	11	9	4	7.00
HymCort	0	1	1	1	0	22	2	14	0	242	2	242	0	2	1	2	0.55
Hamhes	0	29	5	2	0	9	3	4	0	1	2	1	0	2	4	1	0.14
Hym1-5	0	361	49	16	0	748	50	16	0	442	23	6	0	84	26	2	16.66
total	0	461	93	9	0	2843	98	152	0	1222	85	7	0	339	85	4	100

	A. sylvaticus				G. glis				M. agrestis				S. alpinus				
N	8				8				8				11				
	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	
Peziz	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Genea	0	0	0	0	0	0	0	0	0	2	25	1	0	0	0	0	
Bals	0	4	75	2	0	10	38	2	0	5	13	5	0	8	9	8	
Truf	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Tsp	0	0	0	0	0	3	25	3	0	1	13	1	0	0	0	0	
Easp	0	4	38	3	0	0	0	0	0	15	38	6	0	0	0	0	
Egran	0	4	25	4	0	0	0	0	0	0	0	0	0	0	0	0	
Esp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Espimm	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Hyst	0	0	0	0	0	16	38	2	0	263	13	263	0	0	0	0	
Russ1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	9	2	
Russ2	0	2	50	2	0	2	13	2	0	0	0	0	0	1	9	1	
Bol	0	1	25	1	0	6	50	2	0	2	13	2	0	1	9	1	
Ccaes	0	3	50	2	0	0	0	0	0	1	13	1	0	19	18	14	
Oast	0	14	38	4	0	27	13	27	0	0	0	0	0	0	0	0	
Mel	0	1	38	1	0	145	25	73	0	74	50	12	0	53	27	14	
Cort	0	0	0	0	0	0	0	0	0	2	25	2	0	5	9	5	
HymCort	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Hamhes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Hym1-5	0	0	0	0	0	569	38	2	0	36	38	11	0	6	27	4	
total	0	25	100	6	0	571	100	4	0	391	75	12	0	57	73	7	

Electronic Supplementary Material (ESM) 2

The interrelationship of mycophagous small mammals and ectomycorrhizal fungi in primeval, disturbed and managed Central European mountainous forests

Susanne Schickmann, Alexander Urban, Katharina Kräutler, Ursula Nopp-Mayr, Klaus Hackländer

ESM2: Numbers of spores of different taxa of ECM fungi egested by the most frequent small mammal species for each of the four investigated forest types (abbreviations as in ESM1).

Primeval Forest (PF)

	A. flavicollis				M. glareolus				S. araneus				S. minutus			
N	42				52				14				23			
	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps
Peziz	0	3	2	3	0	157	2	157	0	0	0	0	0	0	0	0
Genea	0	2	2	2	0	16	15	3	0	1	7	1	0	0	0	0
Bals	0	6	17	3	0	16	21	3	0	0	0	0	0	4	30	3
Truf	0	4	2	4	0	64	10	3	0	0	0	0	0	0	0	0
Tpub	0	1	7	1	0	2	6	1	0	1	7	1	0	7	26	1
Easp	0	8	14	4	0	69	31	4	0	2	7	2	0	2	9	2
Egran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Emur	0	0	0	0	0	1420	6	24	0	0	0	0	0	7	4	7
Espimm	0	2	5	2	0	0	0	0	0	0	0	0	0	9	4	9
Hneph	0	52	19	3	0	3240	38	112	0	318	29	9	0	3	4	3
Russ1	0	6	5	5	0	149	23	34	0	86	14	64	0	0	0	0
Russ2	0	5	14	1	0	1040	25	3	0	6	14	3	0	4	26	1
Bol	0	320	7	2	0	590	33	55	0	43	7	43	0	0	0	0
Ccaes	0	242	7	59	0	1400	25	40	0	3	7	3	0	0	0	0
Oast	0	2	7	1	0	47	19	4	0	1	7	1	0	2	4	2
Mel	0	1650	36	8	0	3650	52	26	0	12	57	4	0	113	26	2
Cort	0	6	19	1	0	263	27	14	0	9	21	6	0	11	9	6
HymCort	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hamhes	0	290	2	290	0	10	4	6	0	0	0	0	0	0	0	0
Hym1-5	0	109	31	16	0	407	42	16	0	6	14	4	0	84	13	1
total	0	1651	90	9	0	7202	98	137	0	387	71	12	0	135	78	4

Disturbed Area (DA)

	A. flavicollis				M. glareolus				S. araneus				S. minutus			
N	78				2				17				19			
	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps
Peziz	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Genea	0	1	3	1	0	11	100	8	0	0	0	0	0	3	5	3
Bals	0	8	21	3	0	0	0	0	0	7	18	1	0	2	21	1
Truf	0	5	1	5	0	0	0	0	0	0	0	0	0	0	0	0
Tpub	0	1	5	1	0	0	0	0	0	0	0	0	0	4	21	2
Easp	0	69	24	6	0	82	100	44	0	3	12	2	0	0	0	0
Egran	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Emur	0	4	1	4	0	3	50	3	0	0	0	0	0	2	5	2
Espimm	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hneph	0	144	9	2	0	0	0	0	0	0	0	0	0	5	16	1
Russ1	0	15	8	2	0	0	0	0	0	0	0	0	0	0	0	0
Russ2	0	4	26	2	0	0	0	0	0	5	47	3	0	7	37	4
Bol	0	1130	19	27	0	82	100	79	0	3	6	3	0	44	11	23
Ccaes	0	399	4	3	0	8	50	8	0	0	0	0	0	1	11	1
Oast	0	5	9	2	0	0	0	0	0	2	24	1	0	4	11	4
Mel	0	4400	33	5	0	43	100	35	0	2	12	2	0	2	16	2
Cort	0	7	10	4	0	67	50	67	0	2	18	1	0	7	11	5
HymCort	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hamhes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hym1-5	0	530	42	52	0	748	100	421	0	12	29	3	0	10	32	3
total	0	4476	88	10	0	955	100	626	0	13	94	3	0	51	84	6

Managed Forest (MF1)

	A. flavicollis				M. glareolus				S. araneus				S. minutus			
N	11				65				16				32			
	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps
Peziz	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Genea	0	1	9	1	0	43	20	3	0	0	0	0	0	1	3	1
Bals	0	2	9	2	0	17	9	2	0	0	0	0	0	3	16	2
Truf	0	4	9	4	0	71	23	4	0	0	0	0	0	0	0	0
Tpub	0	0	0	0	0	5	2	5	0	2	6	2	0	2	9	2
Easp	0	2	18	2	0	80	22	4	0	0	0	0	0	3	9	1
Egran	0	0	0	0	0	40	3	26	0	0	0	0	0	0	0	0
Emur	0	0	0	0	0	10	5	7	0	0	0	0	0	0	0	0
Espimm	0	0	0	0	0	2	2	2	0	9	6	9	0	0	0	0
Hneph	0	0	0	0	0	3040	37	57	0	207	25	4	0	2	6	2
Russ1	0	21	18	12	0	97	22	5	0	31	6	31	0	5	6	3
Russ2	0	0	0	0	0	1200	25	3	0	7	25	4	0	4	6	3
Bol	0	110	18	56	0	1100	45	12	0	13	13	12	0	3	3	3
Ccaes	0	1860	18	931	0	22	8	3	0	0	0	0	0	0	0	0
Oast	0	5	9	5	0	1010	29	5	0	31	6	31	0	335	16	6
Mel	0	239	64	8	0	5000	71	442	0	387	56	6	0	319	34	4
Cort	0	40	27	2	0	900	42	21	0	1	6	1	0	8	9	4
HymCort	0	1	9	1	0	22	3	14	0	242	6	242	0	2	3	2
Hamhes	0	13	9	13	0	0	0	0	0	0	0	0	0	0	0	0
Hym1-5	0	2	27	1	0	1900	57	21	0	35	19	7	0	13	34	2
total	0	1868	100	8	0	6412	98	666	0	913	81	14	0	339	88	4

Managed Forest 2 (MF2)

	A. flavicollis				M. glareolus				S. araneus				S. minutus			
N	28				48				19				15			
	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps	min	max	%ps	mps
Peziz	0	8	4	8	0	64	2	64	0	0	0	0	0	0	0	0
Genea	0	0	0	0	0	2	4	2	0	1	5	1	0	0	0	0
Bals	0	17	32	2	0	30	44	3	0	4	26	1	0	3	40	2
Truf	0	1	4	1	0	6	4	4	0	0	0	0	0	0	0	0
Tpub	0	3	18	1	0	1	2	1	0	1	11	1	0	1	13	1
Easp	0	34	21	11	0	48	21	4	0	0	0	0	0	2	13	2
Egran	0	2	4	2	0	19	6	10	0	6	16	3	0	0	0	0
Emur	0	3	11	1	0	0	0	0	0	1	5	1	0	0	0	0
Espimm	0	5	7	3	0	132	10	3	0	0	0	0	0	0	0	0
Hneph	0	6	4	6	0	1500	13	2	0	1	5	1	0	2	7	2
Russ1	0	0	0	0	0	14	2	14	0	0	0	0	0	0	0	0
Russ2	0	3200	25	3	0	740	31	24	0	5	26	2	0	57	53	4
Bol	0	0	0	0	0	6	6	5	0	0	0	0	0	0	0	0
Ccaes	0	0	0	0	0	13	4	7	0	0	0	0	0	1	13	1
Oast	0	2	18	1	0	1090	25	34	0	9	21	2	0	3	7	3
Mel	0	3	25	1	0	280	19	2	0	7	16	6	0	3	7	3
Cort	0	1	4	1	0	6	6	4	0	1	5	1	0	0	0	0
HymCort	0	1	4	1	0	3	4	3	0	0	0	0	0	0	0	0
Hamhes	0	1	4	1	0	0	0	0	0	0	0	0	0	0	0	0
Hym1-5	0	370	25	3	0	152	23	2	0	7	11	4	0	4	7	4
total	0	3205	89	9	0	1832	88	23	0	13	79	4	0	57	80	5

2. DNA extraction

Comparison of extraction methods applicable to fungal spores in faecal samples from small mammals
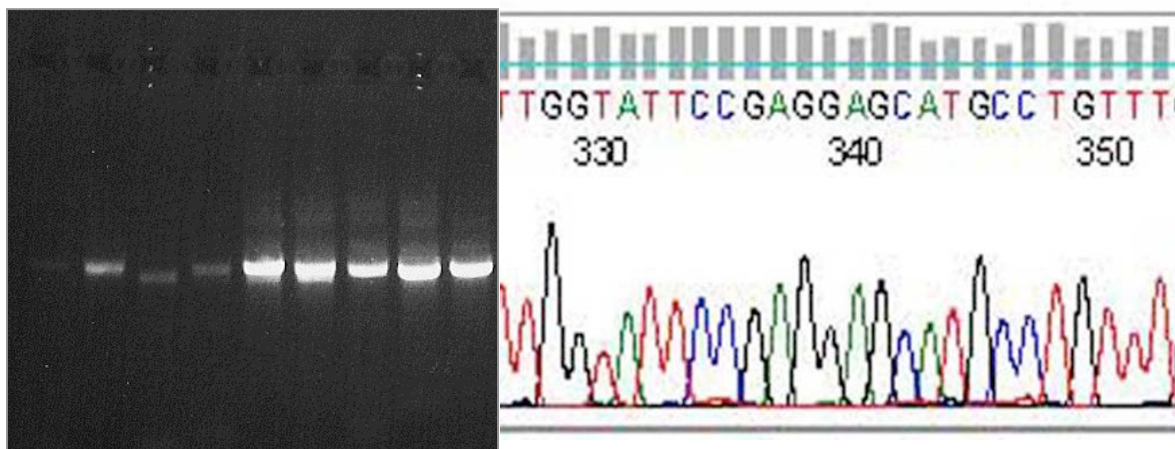
Results of PCR with extracted DNA on Agarose gel (left) and part of the obtained DNA sequence of *Chamonixia caespitosa* (right)

Comparison of extraction methods applicable to fungal spores in faecal samples from small mammals

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Schickmann S., Kräutler K., Kohl G., Nopp-Mayr U., Krisai-Greilhuber I., Hackländer K. & Urban A. (2010) Comparison of extraction methods applicable to fungal spores in faecal samples from small mammals. – Sydowia 63(2): 237–247.

Mycophagy is often underestimated as dietary strategy, but forms an important part of the webs of life, especially in forest ecosystems. The identification of consumed fungal species is crucial to gain more knowledge about food web structures. DNA based methods are the way of choice to overcome limitations of species determination by microscopic analysis. DNA extraction from fungal spores in faecal samples requires specific methodology, due to the resistance of fungal spores and due to the properties of the faecal matrix.

We tested two open source extraction buffers, as well as one widely used commercial extraction kit in combination with a mechanical disruption procedure optimised for fungal spores in faecal samples. The efficiency of the DNA extraction was assessed by comparing DNA yield after amplification by PCR with fungus-specific primers. All three protocols were successful in extracting amplifiable fungal DNA. The CTAB protocol yielded the highest amounts of DNA after PCR and gave the most constant results, but the differences among protocols were not significant. The establishment of reliable protocols for DNA extraction from small quantities of fungal spores in faecal samples paves the way for high resolution non-invasive studies in the dietary ecology of wild-living mammals.

Keywords: mycophagy, dietary ecology, DNA isolation, environmental microbiology,

Today we know that many forest dwelling animals, in particular small mammals, deer, wild boar, and many invertebrates use a food source, which has often been neglected due to its ephemerality and cryptic nature. This source are fungi (Cázares & Trappe 1994, Johnson 1996, Reddel *et al.* 1997) of different phylogenetic and ecological groups, many of them forming a key part of forest ecosystems, the "wood wide web" (Fogel & Trappe 1978, Carey 1999). The relationship of animals and fungi has been analysed more intensely in recent years (Shchipanov *et al.* 2003, Hanya 2004, Hanson *et al.* 2006), but detailed assessments of fungal species consumed by animals remain rare for

temperate European forests. Blaschke & Bäumler (1989) and Bertolino *et al.* (2004) investigated small mammal mycophagy in Central Europe, but they focused on single species only and did not complement microscopic analysis of faecal samples with DNA based methods. Thus, the degree of mycophagy of most European small mammals as well as the majority of fungal species consumed remains unknown.

The diversity of fungi consumed by different animal species can be investigated in two ways: (1) taking faecal samples and preparing microscopic slides, then counting and identifying fungal spores with a light microscope (Cázares & Trappe 1994, Colgan III *et al.* 1997, Vernes & Dunn 2009), or (2) taking faecal samples, extracting total DNA, amplifying selected gene fragments (`barcodes´) of fungal DNA with specific primers and applying strategies to obtain DNA fragments which can be sequenced (Kuske *et al.* 1998, Cubero *et al.* 1999, Manian *et al.* 2001).

Both approaches have their advantages and drawbacks. Obtaining faecal samples is non-invasive and usually the easiest part as small mammals are easily live-trapped and faeces from larger mammals can be directly obtained from their habitats. Preparation of microscopic slides is not difficult, neither, but identification of fungal spores in the faecal matrix as well as classification at the supra-generic, generic or even species level and the quantification of spores from mixed species samples requires expertise and thorough working (Colgan III *et al.* 1997). Spores can be detached from organic debris present in the faecal pellets by thorough homogenisation of the samples and adequate dilution. Keys and monographs of fungal genera (Castellano *et al.* 1989) assist in the microscopical determination of fungal taxa. The direct observation method requires time and careful observation (Castellano *et al.* 1989), as the reproducible quantification requires a sufficient number of observations of random fields of view in the microscope, but is the ideal way to record the diversity of fungal species and the proportions of spore types present in the sample. However, given the diversity of potentially ingested fungal species and the wide range of spore sizes, errors due to overlooking small or inconspicuous spores and misidentifications can not be excluded.

DNA based methods promise to provide a more objective procedure for the assessment of the diversity of ingested fungi and, potentially, accurate identification at the species level. Despite the widespread routine use of DNA extraction techniques, the optimal recovery and purification of fungal DNA from faecal samples remains challenging, because: (1) The available fungal DNA is packed up in the spores, well protected to survive gut passage (Trappe & Maser 1976, Colgan III & Claridge 2002) . Therefore, extraction of DNA requires the disruption of spore cell walls. In order to provide a representative analysis, spore wall disruption needs to be effective for all target species. (2) Low quantities of target DNA require minimization of losses during

extraction. (3) Faecal samples are potentially rich in inhibiting substances or DNA degrading components (DNAses, food components, etc.) of various origins, so these need to be efficiently inactivated to avoid loss of target DNA (Rossen *et al*. 1992, Eggert *et al*. 2005).

Thus, a reliable, inexpensive, and easy to use method for extraction of fungal DNA from faecal samples could boost knowledge on mycophagy of animals and shed light onto the feeding ecology of animal species, species connections, fungal distribution, and possibly also related conservation issues.

Up to date, researchers can choose from a variety of commercially available DNA extraction kits for fungal DNA as well as from a galore of other extraction protocols (e.g. Kuske *et al*. 1998, Cubero *et al*. 1999, Schwarzott & Schüßler 2001), but comparisons between any of them remain rare. Due to the variety of approaches it can be difficult to find the most suitable method available for a particular research question.

We therefore attempt to provide another piece of the puzzle by comparing a popular commercially available DNA extraction kit with two open source extraction protocols. The results can be of significance to any researcher, who wants to assess the feeding ecology and mycophagy of more than one animal species or a species community at the same time, since our favoured method is applicable to and successfully used for different small mammal species with various feeding habits.

Materials and methods

Sampling procedure and storing

We live trapped small mammals according to international standards (Kirkland Jr. 1998, Powell & Proulx 2003, Gannon & Sikes 2007) and obtained faecal samples from each newly captured animal by taking faecal pellets from the traps. We collected the faecal material during five live trapping sessions in 2006 and 2007 in the Dürrenstein Wilderness Area (Austria). Upon collection, samples were transferred into Eppendorf reaction tubes (1.5 mL) filled half with silica gel beads for rapid drying and storage. For further analysis (microscopic and DNA-based), we separated pellets from coarse plant material as well as from silica beads, added 600 µL distilled water and homogenized them mechanically with a conical pistil after short soaking in the Eppendorf reaction tubes. We kept aliquots of the resuspended samples frozen at –20 °C for DNA analysis.

Samples

We selected 16 faecal samples from six species of small ground dwelling mammals (*Myodes glareolus* Schreber 1780, *Apodemus flavicollis* Melchior 1834, *Microtus agrestis* Linnaeus 1761, *Glis glis* Linnaeus 1766, *Sorex araneus* Linnaeus 1758 and *Sorex minutus* Linnaeus

1766) for the comparison, based on amount of fungal spores (spore numbers ranging from 0.5 to 72.7 spores per field of view), but randomly chosen regarding collection time and sample size (total dry weights between 10 mg and 70 mg) (Table 1). We thoroughly resuspended samples by vortexing and divided each sample into three aliquots of 200 µL each (pipetting 100 µL twice using a cut 200 µl pipette tip), to ensure the best possible equality of the aliquots.

Tab. 1. – Faecal samples used for comparison of extraction methods. Spores per 50fov = Number of fungal spores counted in 50 random fields of view (= fov) with 400× magnification, Fluorescence signal = intensity of band with correct size range as calculated with ImageJ, GR – 1 kb GeneRuler; mos = months.

No.	Spores per 50fov	Small mammal species	Collection and storage time	Fluorescence signal		
				AnDNA	CTAB	Kit
1	280	Myodes glareolus	Oct 2007, 30 mos	1279.31	12431.41	18001.21
2	311	Microtus agrestis	Aug 2007, 32 mos	546.09	14500.38	8805.92
3	3633	M. glareolus	May 2007, 32 mos	10138.12	11548.82	11684.36
4	1552	M. glareolus	Aug 2007, 32 mos	15516.23	11333.48	11126.24
5	29	Sorex minutus	Oct 2007, 30 mos	2949.88	12381.31	11219.12
6	1127	M. glareolus	Aug 2007, 32 mos	9964.60	11309.14	2288.95
7	631	M. glareolus	Aug 2007, 32 mos	19940.20	17758.33	10967.65
8	596	Glis glis	Aug 2007, 32 mos	8484.17	868.40	461.16
9	1140	M. glareolus	Oct 2007, 30 mos	572.09	6328.49	3534.23
10	389	M. glareolus	May 2007, 32 mos	12999.39	15329.12	13140.46
11	381	M. glareolus	Oct 2006, 42 mos	3874.40	5940.25	5439.18
12	380	Apodemus flavicollis	Oct 2006, 42 mos	4894.59	3688.23	6053.76
13	320	A. flavicollis	Oct 2006, 42 mos	4039.25	3770.23	5530.13
14	217	Sorex araneus	Aug 2007, 32 mos	4704.47	8051.61	3721.00
15	39	M. glareolus	Jul 2006, 45 mos	144.95	112.95	72.95
16	27	A. flavicollis	Oct 2006, 42 mos	6908.63	13743.26	7525.10
GR	mean	500 bp band			1316.47	

Extraction kit and buffers

The tested commercial kit was the QIAamp DNA Stool Mini Kit (Catalogue no. 51504, No. of preps: 50). We followed the manufacturers protocol for extraction, but added three scoops of fine quartz sand (SIGMA, Catalogue no: S-9887) and five glass beads (diameter 2–3 mm) during the initial lysis steps.

We compared the QIAamp kit with one commonly used extraction buffer for fungal DNA – CTAB (Cubero *et al.* 1999, Izzo *et al.* 2005, Zhang *et al.* 2006) and one very simple buffer established for extracting ancient DNA from paleontological samples – AnDNA (Rohland & Hofreiter 2007).

The CTAB extraction buffer consisted of 0.01 M Tris, pH 8.0, 3 M NaCl, 0.02 M EDTA pH 8.0, and 0.5 M CTAB (Cetrimonium bromide), with 2 % (wt/vol) PVP (polyvinylpyrrolidone), and 0.5 % (vol/vol) ß-

mercaptoethanol added before use. The AnDNA buffer is composed of 400 mM EDTA pH 8.0 and 0.5 mg/mL proteinase K in distilled water.

Extraction protocol (CTAB, AnDNA)

We resuspended each sample aliquot in 600 µL extraction buffer in a 2-mL tube and added 3 scoops of quartz sand as well as five glass beads (diameter 2–3 mm). After sealing with parafilm, we placed the tubes in the grinding mill (Retsch, MM301, Düsseldorf, Germany) and shook them for 20 min at 30 Hz (maximum frequency). Finally, we checked all samples microscopically for the degree of disruption of the spore walls.

We then incubated the samples for one hour in an Eppendorf Thermomixer compact (Eppendorf, Hamburg, Germany) at 55 °C and 800 rpm, transferred the supernatant into a new 1.5-mL tube after centrifugation for 1 min, and added an equal volume chloroform-iso-amylalcohol (24:1). After vortexing, we centrifuged samples for 10 min. We transferred the upper phase into a new 1.5-mL tube and added 1/10 volume of 3 M sodium acetate and 2/3 volume of isopropanol to precipitate DNA.

We pelleted DNA by centrifugation for 30 min after incubation for 5 min at room temperature, discarded the supernatant, and washed the pellet twice with 100 µL 70 % EtOH and centrifuged for 10 min. Subsequently we let the pellet air-dry at 40 °C and dissolved it in 50 µL 10 mM Tris-HCl (pH 7.5) at 55 °C and 1000 rpm in an Eppendorf Thermomixer compact (Eppendorf, Hamburg, Germany). We carried out all centrifugation steps at top speed (13.000 rpm ~ 16.100 g) using an Eppendorf Centrifuge 5415D (Eppendorf, Hamburg, Germany).

DNA purification

We purified the DNA solution of the 32 samples extracted with the open source buffers using the Invisorb® Spin PCRapid Kit (Invitek, Berlin, Germany) according to the manufacturer's protocol with slight modifications as recommended by the manufacturer – we increased centrifugation time at step two and four to 1.5 min and 10 min, respectively. We increased the volume of the elution buffer in step five to 40 µL and extended the incubation time at room temperature with elution buffer to 10 min. DNA extracts were stored at –20 °C.

Detection of DNA and PCR conditions.

We checked DNA contents of genomic DNA extracts and PCR products by loading 5 µL onto an 1 % agarose gel (0.5 x TAE buffer) stained with GelRed™ (Biotium, Hayward, CA, USA). We ran the gel electrophoresis for 25 min at 90 V, using the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) for approximate size determination.

We used the fungi specific primer pair ITS1F/ITS4 (ITS1F: CTT-GGTCATTTAGAGGAAGTAA; ITS4: TCCTCCGCTTATTGATATGC, typical amplicon size: 650 bp) for amplification of fungal DNA by PCR. ITS1F binds close to the 3'-end of the nuclear 18S rDNA (nrSSU; Gardes & Bruns 1993), whereas ITS4 (White *et al*. 1990) anneals to a conserved region close to the 5'-terminal part of the nuclear 28S ribosomal DNA (nrLSU). Using the extracted samples as template DNA, we amplified a fragment of fungal DNA fragments ranging approximately between 550 bp and 700 bp in size. We prepared the following amplification mixture: 1X Taq Buffer + NH_3SO_4 (Fermentas), 2.5 mM $MgCl_2$ (Fermentas), 200 mM each dNTP, 0.8 µg/µL BSA, 1 % DMSO, 0.5 µM each primer, 2 µL Taq polymerase recombinant (Fermentas, Catalogue No: EP0404, 1 u/mL), and 1 µL of each extracted sample solution (undiluted), and distilled water to a total volume of 25 µL. PCR cycling parameters were initial denaturation 120 s at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 54 °C, 90 s at 72 °C, and final elongation for 10 min at 72 °C. To minimize the influence of external factors potentially affecting PCR efficiency, we amplified the DNA in a single PCR run, using one master mix and one PCR plate for all samples.

Evaluation criterion and statistics

We used PCR efficiency as criterion instead of genomic DNA yields, because (1) genomic DNA yields were generally very low, precluding a precise quantification after extraction, (2) stool sample genomic DNA is of various origins, the proportion of fungal DNA is a priori unknown, and (3) PCR efficiency is most important for all downstream applications.

We quantified PCR amplified DNA yields based on agarose gel band intensity using the open source program ImageJ (Rasband 1997). We applied square root + inverse transformation to the fluorescence signal data and inverse transformation to the No of spores to obtain a normally distributed data set. We examined potential differences in extraction efficiency between the three protocols applying (1) one-way ANOVA with repeated measurements (rmANOVA, model: transformed fluorescence signal ~ method + Error (sample/method)) with methods being AnDNA, CTAB and Kit and (2) two-way ANOVA (model: transformed fluorescence signal ~ method * transformed No of spores) to account for potential correlations between spore numbers and PCR efficiency. We used the statistical environment R (R Development Core Team 2011) for all calculations.

Results

We achieved an extraction/PCR efficiency of nearly 100 %, amplifying fungal DNA from 15 out of 16 samples in sufficient quantities. The extraction of fungal DNA from faecal samples of small mammals

was successful even after more than three years of storage at –20°C (Tab. 1). Sample no 15 did not show bright bands on the agarose gel (Fig. 1a), but the quantification program ImageJ did detect small amounts of DNA of the correct size (Tab. 1). However, as very small amounts of amplified DNA are generally not sufficient for downstream applications such as dye terminator sequencing, we classified this sample as failed and excluded it from statistical calculations.

Calculation of mean and median fluorescence signal value (AnDNA = 6684.8 / 4799.5, CTAB = 9318.5 / 11321.3, Stool DNA Kit = 7473.2 / 6789.4) and visual method comparison showed CTAB to yield the highest amounts of DNA after PCR, but neither one way rmANOVA (P = 0.548) nor two-way ANOVA (P(method) = 0.374, P(NoOfSpores) = 0.139, P(method:NoOfSpores) = 0.743) did reveal significant differences.

We determined the number of fungal spores prior to extraction, but there is no obvious relationship between spore numbers in 50 random fields of view (400x magnification) and fluorescence signal after PCR for any extraction method (Fig. 1b). Nevertheless, we could visualise the lower average DNA yield for samples extracted with AnDNA buffer and the QIAamp kit compared to the CTAB buffer and the high scatter of the individual performances.

Discussion

All three tested extraction methods can be regarded as quite reliable, in most cases yielding DNA extracts suitable for the PCR amplification of nuclear ribosomal ITS sequences, a region commonly used in DNA based identification of many fungal groups, including ectomycorrhizal fungi (Kõljalg *et al.* 2005). Furthermore, sequencing of the obtained PCR fragments revealed fungal species observed in the microscope prior to extraction, confirming the suitability of the extraction protocols.

Despite the high dispersion of the results, certain differences between the methods could be observed: AnDNA buffer gave the weakest and most variable results particularly when low numbers of fungal spores were observed in the sample. Fungal DNA fragments could be amplified in sufficient amounts from 75 % of the samples extracted with this protocol. The QIAamp DNA Stool Mini Kit led to satisfactory results in 80 % of the samples, and seemed to achieve more constant results in PCR efficiency than the AnDNA extraction protocol. The CTAB method, which is a common procedure for fungal DNA extraction effective for environmental samples (Izzo *et al.* 2005) shows the most stable results for small mammal faecal samples, especially for those with low numbers of spores. It failed only once in sample 15, but this sample failed with the other two methods as well. However, successful PCR amplification of fungal DNA from samples with few spores
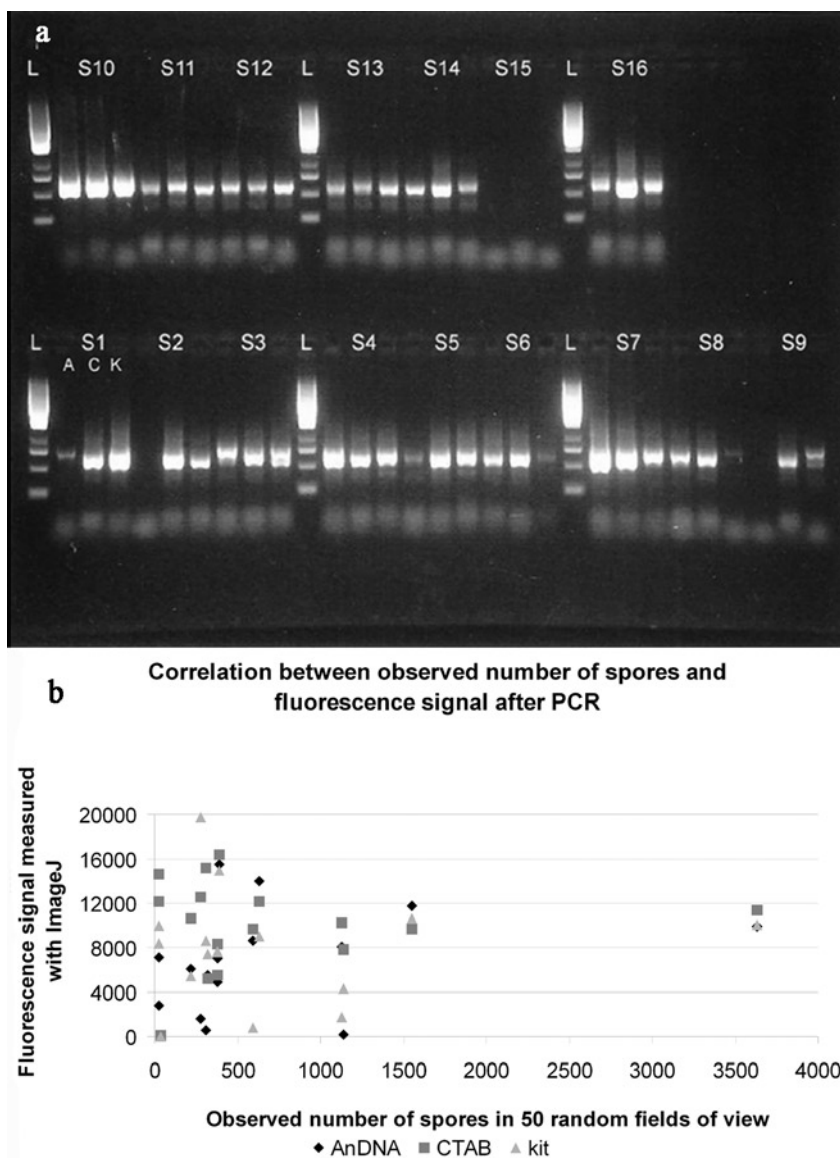
Fig. 1 – (**a**) Agarose gel stained with GelRedTM showing all 16 faecal small mammal samples after fungal DNA extraction and PCR with ITS1/ITS4 primers. Amplification using the same primers but no template (negative control) resulted in no PCR product (data not shown), L: 36 GeneRuler 1kb (Fermentas), S1–S16: sample numbers, A-AnDNA buffer, C-CTAB buffer, K37 QIAamp DNA Stool Mini Kit; same order for every sample. (**b**) Fluorescence signal of the extracted samples as calculated with ImageJ in relation to the total number of fungal spores observed in 50 random fields of view with 400× magnification.

244

frequently contain a high proportion of sequences from yeast genera such as *Cryptococcus* and *Rhodotorula*, and rare target fungi can be missed, even if a larger pool of clones is analysed (Urban *et al.*, unpub.). Therefore, it is more advisable to use samples rich in target spores. Possibly, the high proportion of positive PCR results from samples with few spores when using the CTAB and QIAmp protocols is due to efficient lysis of yeast cells, which might also explain the absence of a correlation between numbers of larger fungal spores (yeast cells were not counted) and DNA amplicon yields.

Selection of DNA polymerase seems to be essential, too. The taq polymerase chosen was compatible with all three extraction methods, which was not the case with certain other DNA polymerases, which were tested randomly in the earlier phases of protocol development. PCR with DNA extracts obtained with the commercial stool DNA extraction kit was least sensitive to DNA polymerase choice, suggesting that this method provides the lowest level of PCR inhibitors, and may be the best choice in case of very sensitive downstream applications.

There may be potential for further improvement of DNA extraction from fungal spores in faecal samples. The inclusion of additional washing and/or concentrating steps prior to the extraction procedure might further reduce PCR inhibitors and raise the relative proportion of target DNA. However, we found fungal spores distributed across different phases in all solutions to discard after initial fractionation and additional washing steps, so we prefer to work with the whole stool samples in order not to loose any information on certain (possibly rare) species.

As we suppose that any DNA contained in ingested fungal hyphae is likely to be too degraded for successful amplification, the disruption of the spore walls is crucial for extracting fungal DNA from spores out of faecal samples. The successful mechanical spore disruption applied in all extraction protocols contributed to the reliability of the three tested methods and is probably essential regardless of the extraction protocol chosen afterwards. The established method resulted in the identification of more than 30 species of fungi from small mammal scats (Urban *et al.*, unpubl.) and may help other scientists to shed more light onto the field of mycophagy and its role in the webs of life.

Acknowledgments

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3. Inoculation experiments

a. Root exudates and their effect on *Striga* germination

Stimulation of *Striga* seeds by root exudates of AM host and nonhost plants cannot be linked with their host status to AM fungi

Inoculated beech (*Fagus sylvatica*) and fir (*Abies alba*) seedlings

Short Communication

The arbuscular mycorrhizal host status of plants can not be linked with the *Striga* seed-germination-activity of plant root exudates

Der Wirtspflanzenstatus der vesikulären-arbuskulären Mykorrhiza korreliert nicht mit der *Striga*-Keimungsaktivität von Pflanzenwurzelexsudaten

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Abstract

Root exudates from sorghum, a *Striga* and arbuscular mycorrhizal fungal (AMF) host plant, and a number of *Striga* non-host plants which are AM host or AM non-host plants were collected and their effect on seed germination of *Striga hermonthica* was tested. *Striga* seeds germinate exclusively in presence of strigolactones, thus, *Striga* seeds germination is an indicator for the presence of strigolactones. Strigolactones are also thought to be essential signals (branching of AMF) for a successful root colonization by AMF. Root exudates of AM host plants stimulated *Striga* seed germination or showed no effect, whereas root exudates of AM non-host plants never showed an effect on *Striga* seed germination. This means that *Striga* seed germination stimulation by compounds in root exudates can not necessarily be linked with the host and nonhost status of plants to AMF. This absence of an effect on *Striga* seed germination of root exudates of AM host plants can be linked i) either with different levels of strigolactones in the root exudates of different AM host plants or ii) with the fact that not the same compounds are involved in *Striga* seed germination and in signaling during the establishment of the AM symbiosis.

Key words: arbuscular mycorrhiza, germination assays, *Striga*, strigolactones, root exudates

Zusammenfassung

Wurzelexsudate von Sorghum-Hirse, einem Wirt der parasitischen Samenpflanze *Striga* und von arbuskulären Mykorrhizapilzen (AMP), sowie einer Reihe von Nichtwirten von *Striga*, die entweder Wirte oder Nichtwirte von AMP sind, wurden gesammelt und ihre Wirkung auf die Samenkeimung von *Striga hermonthica* untersucht. *Striga*-Samen keimen nur in Gegenwart von Strigolactonen, weshalb ihre Keimung als Indikator für Strigolactone dient. Strigolactone gelten daneben als essenzielle Signale für eine erfolgreiche Wurzelbesiedlung durch AMP. Wurzelexsudate von AMP-Wirten stimulierten zum Teil die Keimung von *Striga*-Samen, während Wurzelexsudate von AMP-Nichtwirten niemals die Samenkeimung anregten. Dies bedeutet, dass die Stimulierung der Samenkeimung bei *Striga* durch Komponenten von Wurzelexsudaten keinen Rückschluss auf den Wirtsstatus von Pflanzen gegenüber AMP erlaubt. Die fehlende Wirkung der Wurzelexsudate von AMP-Wirten auf die Samenkeimung von *Striga* kann entweder auf i) der unterschiedlichen Konzentration von Strigolactonen in den Wurzelexsudaten von AMP-Wirtspflanzen beruhen oder ii) darauf, dass nicht dieselben Exsudatkomponenten für die Samenkeimung und als essenzielle Signale für die AMP-Besiedlung von Wurzeln verantwortlich sind.

Stichwörter: arbuskuläre Mycorrhiza, Keimungstests, *Striga*, Strigolactone, Wurzelexsudate

1   Introduction

Arbuscular mycorrhizal fungi (AMF) are a group of obligate mutualistic fungi that associate with roots to form the arbuscular mycorrhizal symbiosis. Strigolactones are apocarotenoids exuded by roots in extremely low concentrations which stimulate seed germination of the parasitic weeds *Striga* and *Orobanche* (Bouwmeester et al. 2007; Steinkellner et al. 2007) and act as signaling molecules for the establishment of the AM symbiosis due to their activity on AM hyphal branching (Akiyama et al. 2005). Recently, strigolactones have been identified as a new class of plant hormones regulating shoot branching, suggesting that all plant species produce these compounds (Gómez-Roldan et al. 2008; Umehara et al. 2008). However, an activity on AM hyphal branching of root exudates due to strigolactones has been reported only for AM host plants of but not for AM nonhost plants (Buée et al. 2000; Nagahashi and Douds 2000).

Germination of *Striga* or *Orobanche* seeds after root exudate application clearly indicates the presence of strigolactones in the exudates. Data on the effect of root exudates on the germination of *Striga* and *Orobanche* seeds are available from a number of plants from different studies, however, the experimental set-up in these studies is always varying e.g. different seed patches are tested and/or the collections of root exudates differed. Thus, comparing the data from different studies on the effect of root exudates from different plants on *Striga* and *Orobanche* seed germination and concluding from these data on the level of seed germination-stimulating compounds in root exudates is critical. To our knowledge there is

only one study with *Orobanche* (Westwood 2000), but none with *Striga*, where the effect of compounds released by roots of different plants on seed germination was directly compared, thus giving indications on differing levels of seed germination-stimulating compounds depending on the tested plant.

In the present study, by testing the effect of root exudates from sorghum (a *Striga* and AMF host plant) and a number of *Striga hermonthica* non-host plants which are AM host or AM non-host plants we wanted to obtain data on the presence of *Striga* seed-germination-stimulating compounds in root exudates of *Striga* non-host plants and their possible involvement in the determination of the AM status of AM host and non-host plants.

2 Materials and methods

2.1 Plant material and collection of root exudates

The root exudates of a range of plants (see Table 1) were tested. Apart from the tree species, seeds of all plant species were surface sterilized in 1% aqueous NaOCl for 5 min and pregerminated in perlite for 5 days. Thereafter four plantlets of each plant species were transferred to pots filled with autoclaved (for 20 min at 121°C) substrate: a mix of arable soil, fine sand (< 2 mm) and expanded clay in the ratio 1:1:1 v/v/v in pots.

Seeds of *Populus tremula* and *Pinus sylvestris* were surface sterilized in H_2O_2, rinsed several times with sterile water and germinated thereafter on agar. *Quercus robur* seeds were germinated in moistened perlite. Plantlets were transferred to pots containing autoclaved peat.

Salix caprea plants were obtained from tissue cultures. Plantlets were transferred to pots with clay granules (SERAMIS; Mars, Inc., Hackettstown, NJ, USA) as a substrate.

Fagus sylvatica seeds were surface sterilized in H_2O_2, rinsed several times with sterile water and germinated thereafter in wet sand at 4°C. Plantlets were transferred to pots to an autoclaved mixture of seedling peat substrate, sand and perlite (1:1:1).

Four replicate pots per plant species were set up. All pots were placed in the greenhouse at the Universität für Bodenkultur in spring 2007. The seedlings were watered regularly on a daily basis. Three weeks after emergence (except trees: *P. tremula* and *Q. robur*: 2 months; *P. sylvestris* and *F. sylvatica*: 3 months; *S. caprea*: 4 months), the roots of all the seedlings were washed free of adjoining substrate and placed in beakers of varying sizes depending on the volume of each root system. Distilled water was added to the beakers to barely cover each root system. The beakers were wrapped with aluminium foil to simulate dark conditions as previously in the substrates. The seedlings were placed under these conditions for 24 hours. Subsequently, each seedling was removed; the root system cut off and blotted with tissue paper then weighed with an electronic balance. The solution in each beaker was diluted with distilled water to the ratio 1 g root fresh weight to 10 ml of water. This solution was used as fresh root exudates on pre-conditioned *S. hermonthica* seeds.

2.2 Preconditioning Striga seeds

Ten milligrams of *S. hermonthica* seeds harvested from sorghum as host at Maroua (latitude 10° 30' and 11° N and longitude 14° and 14° 30' E) Cameroon in October 2005) were surface sterilized in a laminar flow in 2% NaOCl (for

Table 1: Effect of root exudates of AM host and nonhost plants on the germination of *Striga* seeds

Plant species	Family	Scientific name	Germination (%)	Mycorrhizal status
Control	GR24 (10-7M)		53.7 a	
CK60B sorghum	Gramineae	*Sorghum bicolor*	32.1 b	AM
Barley	Gramineae	*Hordeum vulgare*	1 e	AM
Cucumber	Cucurbitaceae	*Cucumis sativus*	25.5 b	AM
Zucchini	Cucurbitaceae	*Cucurbita sp*	0 f	AM
Basil	Lamiaceae	*Ocimum basilicum*	0 f	AM
Leek	Alliaceae	*Allium porrum*	0 f	AM
Carrot	Apiaceae	*Daucus carota*	0 f	AM
Tomato	Solanaceae	*Solanum lycopersicum*	0 f	AM
Common Bean	Fabaceae	*Phaseolus vulgaris*	15.5 c	AM
Soybean	Fabaceae	*Glycine max*	2.3 d	AM
Pea	Fabaceae	*Pisum sativum*	2.2 d	AM
Lupine	Fabaceae	*Lupinus spp*	0 f	non-AM
Radish	Cruciferae	*Raphanus sativus*	0 f	non-AM
Green cabbage	Cruciferae	*Brassica oleracea*	0 f	non-AM
Kohlrabi	Cruciferae	*B. oleracea*	0 f	non-AM
Spinach	Amaranthaceae	*Spinacia oleracea*	0 f	non-AM
Tartary buckwheat	Polygonaceae	*Fagopyrum tataricum*	0 f	non-AM
Buckwheat	Polygonaceae	*F. esculentum*	0 f	non-AM
Poplar	Salicaceae	*Populus tremula*	0 f	AM/EM
Willow	Salicaceae	*Salix caprea*	0 f	AM/EM
Pine	Pinaceae	*Pinus sylvestris*	0 f	EM
European beech	Corylaceae	*Fagus sylvatica*	0 f	EM
Oak	Fagaceae	*Quercus robur*	0 f	EM

AM = arbuscular mycorrhizal host; non-AM = AM nonhost; EM = ectomycorrhizal host. Values followed by different letters are significantly different according to the Student-Newman-Keuls test at 5%.

details see LENDZEMO et al. 2007). After surface sterilization the dry surface sterilized *Striga* seeds were carefully tapped onto 1-cm diameter glass fibre filter paper (GFFP) (about 50 to 80 seeds per disc to ease counting). The Petri dishes were sealed with Parafilm, then wrapped in aluminium foil and incubated for conditioning at 28°C in the dark for 21 days.

2.3 Germination test

For germination assays, the preconditioned seeds on the 1-cm GFFP discs were removed from the Petri dishes and dried on tissue paper for 30 minutes at room temperature. The GFFP discs containing seeds were transferred to new lids of 9 cm Petri dishes (5 discs per Petri dish), lined with a filter paper (Whatman® filter paper No. 2) ring wetted with 1 ml of distilled water. 50 µl of the root exudates solution of each seedling or a GR24 solution (10^{-7} M) were added to each GFFP discs containing *Striga* seeds. GR24 is a synthetic strigolactone analogue kindly provided by Prof. B. Zwanenburg (Department of Organic Chemistry; Radboud University, Nijmegen, The Netherlands).

A positive control (50 µl of GR24) and a negative control (50 µl of distilled water per disc) were included. The dishes were sealed with Parafilm and incubated in the dark at 28°C for 48 hours. The Petri dishes were observed under a binocular (stereo) microscope for germination of seeds. Seeds are considered germinated when the radicle comes out of the seed coat.

3   Results

A germination of more than 50% could be observed with the strigolactone analogue GR24 (10^{-7} M) showing that the used *Striga* seeds were viable. Also root exudates of the *Striga* host sorghum and the *Striga*-nonhosts cucumber and common bean showed a clear stimulation of seed germination. Root exudates of soybean, pea and barley only slightly stimulated seed germination, whereas in presence of root exudates of all other tested plants no seed germination could be observed.

4   Discussion

Due to their role as plant hormones regulating shoot branching, strigolactones are thought to be present in all plant species (GÓMEZ-ROLDAN et al. 2008; UEYAMA et al. 2007). When we tested the effect of root exudates of a diverse range of plants on the seed germination of *Striga* we found that root exudates either stimulated *Striga* seed germination indicating the presence of strigolactones or showed no effect.

At first sight the data we obtained with root exudates from AM nonhost plants and their effect on *Striga* seed germination seemed to indicate that in root exudates of AM nonhost plants strigolactones are absent. Comparing the effect of root exudates of AM nonhost and host plants on *Striga* seed germination we found that exudates of none of the tested AM nonhost plants such as radish, green cabbage, kohlrabi (all Brassicacea), lupine and several plants forming ectomycorrhizal associations exhibited a *Striga*-seed germination stimulatory effect. These observations seem to confirm that in root exudates of AM nonhost plants strigolactones are absent and thus, also can not induce AM hyphal branching (BUÉE et al. 2000; NAGAHASHI and DOUDS 2000).

However, these data are in contrast with data obtained in studies with *Orobanche*. Lupine, an AM nonhost plant in the normally mycotrophic leguminoseae, recently has been reported to exude strigolactones which stimulate *Orobanche* seed germination, although strigolactone levels are much lower compared to legumes which are AM host plants (YONEYAMA et al. 2008). WESTWOOD (2000) found *Orobanche* seed germination in presence of *Arabidopsis thaliana*, a member of the

Brassicacea and an AM nonhost plant, although it was reduced by 40% compared to carrot and tobacco and most recently several strigolactones have been identified in root exudates of *A. thaliana* (GOLDWASSER et al. 2008). Moreover, in several studies it has been shown that root exudates of rape and cabbage, both Brassicacea, stimulate the germination of *Orobanche* seeds (JACOBSOHN and LEVY 1986; BENHARRAT et al. 2003), indicating the presence of strigolactones in the root exudates of these AM nonhost plants.

As strigolactones also seem to be present in root exudates of AM nonhost plants which did not exhibit an effect on *Striga* seed germination in our experiment and do not induce AM hyphal branching (BUÉE et al. 2000; NAGAHASHI and DOUDS 2000), *Orobanche* seed germination might be more sensitive to the presence of specific strigolactones than *Striga* seed germination and AM hyphal branching. Our results indicate that the level of strigolactones is reduced in all AM nonhost plants we tested.

Root exudates of all AM host plants tested so far have been shown to exhibit an AM hyphal branching activity (BUÉE et al. 2000; NAGAHASHI and DOUDS 2000), thus indicating the presence of strigolactones. Sorghum is an AM host plant and also a host for *Striga*, thus it was not surprising that in our experiment the highest level of *Striga* seed germination was reached in presence of sorghum root exudates. To our surprise in presence of root exudates from the other tested AM host plants extremely varying effects could be observed. Whereas in presence of cucumber and common bean root exudates intermediate levels of *Striga* seed germination were observed, low levels of germination occurred with barley, soybean and pea and no germination was observed with basil, zucchini, leek, carrot and tomato root exudates. Strigolactones levels even varied extremely in root exudates from member of the same plant family, as high germination levels were observed with sorghum and low with barley (both Gramineae) and intermediate levels were observed with cucumber, whereas no germination occurred with zucchini (both Cucurbitaceae). To our knowledge, these differences in *Striga* seed germination stimulating compounds are not reflected in a different susceptibility of these plants to AMF.

Taken together, our data with root exudates of AM host plants inducing different degrees of *Striga* seed germination could either indicate that the status of plants as AM hosts is not defined by a similar level of strigolactones in their root exudates and/or that different strigolactones are involved in *Striga* seed germination and in signaling during the establishment of the AM symbiosis (branching of AMF).

To summarize, our data indicate that *Striga* seed germination stimulation by compounds in root exudates can not necessarily be linked with the host and nonhost status of plants to AMF, as not only root exudates of AM nonhost plants but also root exudates of AM host plants showed no effect on *Striga* seed germination. This absence of an effect on *Striga* seed germination of root exudates of some AM host plants can be linked i) either with different levels of strigolactones in the root exudates of AM host plants or ii) with the fact that not the same compounds are involved in *Striga* seed germination and in signaling during the establishment of the AM symbiosis (branching of AMF).

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b. Greenhouse inoculation experiments

The mycorrhizae found on the tree seedlings inoculated with small mammal faecal suspension did not reflect the frequency and abundance of ECM fungal spores determined during microscopic identification of fungal spores in small mammal scats.

Ascomycete mycorrhizae (especially *Tuber spp.*) were the dominant types of ectomycorrhizae found on seedlings from beech, spruce and fir across all small mammal species.

Due to heat stress in the greenhouse the number of seedlings was too low for detailed statistical analyses, but visual investigation revealed the predominance of ectomycorrhizae from *Tuber spp.*.

c. Field inoculation experiments

Visual identification of mycorrhizae formed on the tree seedlings planted into sterilized soil in the Rosalia Demonstration Forest plots revealed that there were no mycorrhizae present on the feeder rootlets of the seedlings. This is probably due to the low availability of fungal fruit bodies that is reflected in the significantly reduced degree of mycophagy of the small mammals captured in this area (for detail see chapter 1 of the results).

We can therefore state, that the use of heat sterilized soil and the micromesh covering of drainage holes are efficient for protection of seedlings from fungi occurring in the surrounding soil. Unfortunately, we can not evaluate the vector function of small mammals from this experiment, but we can conclude, that airborne spore transport of mycorrhizal fungi is unsuitable for colonizing "non-mycorrhized" habitats.

Nevertheless, we consider the experimental layout to be suitable to assess the vector role of small mammals, but suggest (1) to repeat it in an area of known higher levels of mycophagy of small mammals and higher availability of fungi, (2) to use a suitable kind of bait attracting small mammals to the bare ground, and (3) to use a mesh width of the wire cages suitable for the regional small mammal community.

4. Small mammal trapping

The small mammal trapping carried out in the Dürrenstein Wilderness Area for the investigation of mycophagy was part of a long term small mammal population monitoring program (Nopp-Mayr *et* al. in prep.).

In the five sessions we live trapped in the Dürrenstein Wilderness Area and in the eight sessions completed in the Rosalia Demonstration Forest we achieved an overall trapping success of 13.08 captures/100 trap nights. The four main small mammal species (Fig. 8) were Yellow necked mouse (*Apodemus flavicollis)*, Bank vole (*Myodes glareolus)*, Eurasian pygmy shrew (*Sorex minutus*), and Common shrew (*S. araneus*). We captured animals of 12 small mammal species, but relative proportions varied between survey areas (Fig. 9).

Fig. 8: The four most frequently captured small mammal species: *Apodemus flavicollis* (top left), *Myodes glareolus* (top right), *Sorex minutus* (bottom left), *S. araneus* (bottom right)
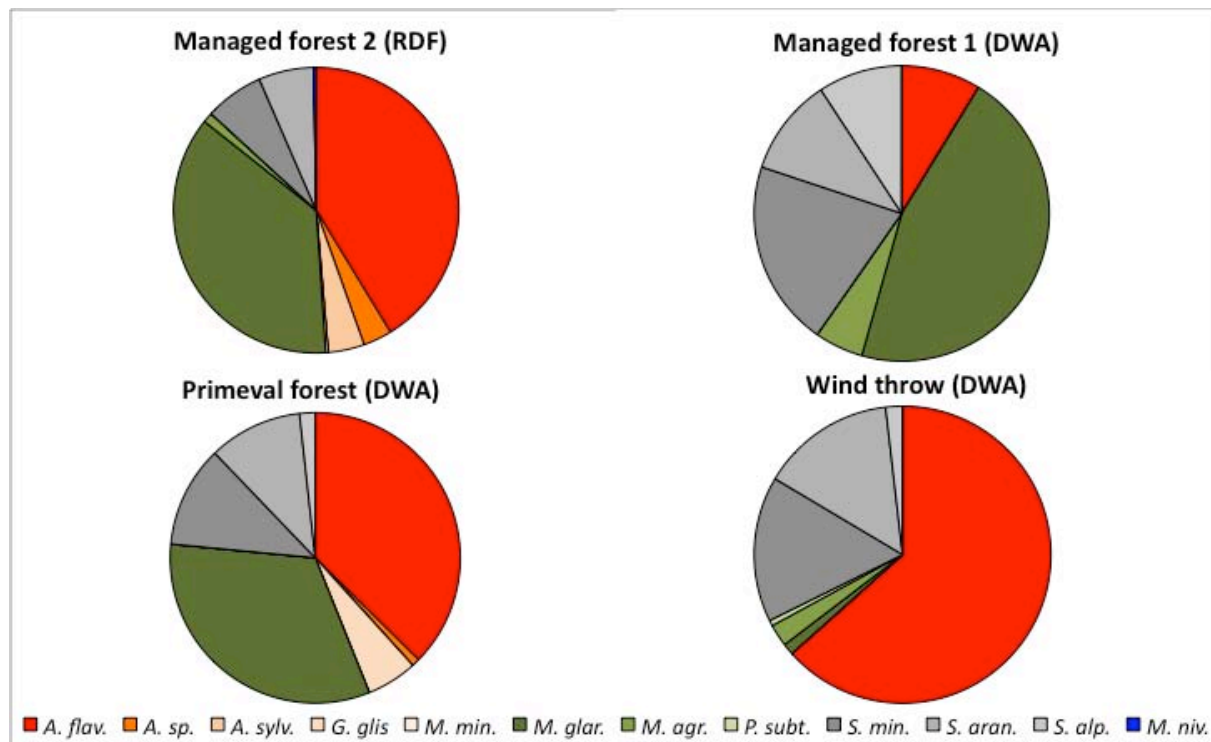
Fig. 9: Small mammal community in the investigated forest areas (*A. flav. – A. flavicollis, A. sylv. – A. sylvaticus, G. glis – Glis glis, M. min. – Micromys minutus, M. glar. – Myodes glareolus, M. agr. – Microtus agrestis, P. subt. – Pitymys subterraneus, S. min. – Sorex minutus, S. aran. – S. araneus, S. alp. – S. alpinus, M. niv. – Mustela nivalis*)

5. Network properties of bipartite mutualistic web

The calculated network indices characterise our bipartite relationship as a nested community, where few more specialised species interact with more generalised species. We found a high redundancy of interactions between the two trophic levels, leading to the assumption that the mycophagist fungi interrelationship in European mountain forests is resilient towards disturbances. The generally low level of specialisation supports this hypothesis. The comparison of mycophagy networks from different ecosystems showed similar levels of the relevant indices for all of them (Table 1).

Table 1: Indices describing network specialisation for three different mycophagy bipartite relationships (* - mycophagy in Austria)

	Network 1	Network 2*	Network 3
Interaction Evenness	0.903	0.897	0.869
Generality	9.767	19.413	16.816
Vulnerability	2.785	6.333	3.029
H_2'	0.271	0.129	0.289
Shannon Diversity	3.475	4.999	4.270

Interaction evenness is a measure for variation in interaction frequencies between different species pairs, with low values (< 0.5) depicting high variation. The values we calculated for

the three given networks lead to the result of low variation in interaction frequencies. Generality and Vulnerability give an estimate of interaction diversity, and therefore gives a measure of the association complexity of the system. The high values of generality and rather low values of vulnerability again show a high level of redundancy of interactions across all three mycophagy networks. H_2' describes complementary specialisation on the community level, so low H_2' values correspond to low complementary or exclusiveness of interactions and therefore suggest a high level of niche overlap and interaction redundancy in all three compared networks (Blüthgen 2010).

The high level of Shannon diversity also depicts a low specialisation of the species within the network for all three compared mycophagy interrelationships (Dormann et al. 2009).

Discussion and Conclusions

Due to the interdisciplinary nature of the investigation a wide assembly of results lead and leads to a better understanding of some of the interweaving processes taking place in forests. Even though the importance of mycophagous animals (and mammals in particular) has been shown in other regions of the world, no research shed light onto the role small mammals play in this regard in European mountain forests so far.

We proofed the vector function of some small mammal species by collecting faecal samples and identifying the fungal spores contained therein. We evaluated the degree of mycophagy of the four most frequent small mammal species in European mountain forests and could show that *M. glareolus* probably is a mycophagist by choice, while *A. flavicollis* and surprisingly also *S. araneus* and *S. minutus* seem to be opportunistic mycophagists. As all of them probably contribute to spore dispersal by endozoochory small mammals in forests should not be reduced to a function of seed predators or "threat of rejuvenation".

We found more than 70 distinct spore types in over 600 small mammal faecal samples and were able to determine ECM fungi, saprophytic fungi as well as plant pathogens. This implies that the role small mammals play in spore distribution might not be limited to fungi forming mycorrhizae with tree roots. It rather seems that the whole cosmos of fungal inhabitants in a forest is at least partly distributed through the dropping of scats.

Nevertheless, we found microscopic determination of fungal species to have some drawbacks. It is rather time consuming when rare fungal spore types are in focus also, it is

error prone if more than one person works at the task, and most important some fungal species can not determined by spore morphology alone.

On the other hand, molecular determination can not be the only way, too, because DNA from rarely occurring fungal spores are not amplified to the same number as more frequent DNA is. So, rare fungal species found in low spore numbers in the samples will probably be overlooked. Furthermore, most small mammal faecal samples contain an array of fungal spores, so that no direct sequencing is possible and also there might be inhibiting substances in the faeces obscuring results. Some fungal spores might even be resistant to grinding so their DNA is not found in the extracted sample.

We therefore suggest a combination of both methods: A microscopical screening (possibly with less fields of view to speed up the investigation) followed by careful mechanical spore disruption, DNA extraction, and DNA amplification with fungal specific primers.

Another important result of our study was the fact, that the mycorrhizae formed on the rootlets of the tree seedlings inoculated with a suspension of small mammal faeces did not resemble the fungal species composition as determined by microscopic analysis of the spores (Urban et al. in prep.). The fungal species forming the mycorrhizae were rarely observed as spores in the small mammal samples.

The field inoculation experiments showed that our study layout was very suitable regarding the preclusion of cross contaminations from the surrounding soil as the sterile seedlings did not show any mycorrhization. For a real evaluation of the vector function of animals areas of primary succession (glacier forefronts, land laid bare by receding floods, etc.) will probably be more informative. Another possibility would be the baiting of the enclosures with sterile soil and seedlings to attract potential vectors.

So concluding we can state, that small mammals seem to play an important role in the spread of a variety of fungal species, even though we could not attribute obligate mycophagy to one of the small mammal species studied. The reason for this might be the unpredictable climate of Central Europe which may lead to high fluctuations in availability of fungal fruit bodies. Another reason might be the diverse feeding habits of the vector species with less pressure on specialisation as might occur in more species rich habitats.

The vector role of insectivorous species has only been started to be understood and more research is needed regarding their role in this specific process.

But one result is very encouraging: The spore composition of animal faeces can also be used to study the distribution and habitat requirements of fungal species as well, especially of hypogeous or inconspicuous species.

If the results of our project will lead to a better understanding of the interweaving forest processes and to the acknowledgement, that small mammals are an important part of these interrelationships, we have not only filled a gap in scientific knowledge, but were also able to transport this message to the people concerned most directly. Because only a healthy forest with its wood wide web and all threads leading to and from it can function properly, be resilient to various kinds of changes and meet all the economic, social and ecological demands of the people living in its vicinity.

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