Digital Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Science and Technology 2534

The Origin and Diversification of Ectomycorrhizal Fungi

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ACTA UNIVERSITATIS
UPSALIENSIS
2025

ISSN 1651-6214
ISBN 978-91-513-2470-8
urn:nbn:se:uu:diva-552928

UPPSALA
UNIVERSITET

Dissertation presented at Uppsala University to be publicly examined in Friessalen, Norbyvägen 16, Uppsala, Wednesday, 28 May 2025 at 09:15 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Håvard Kauserud (Other:University of Oslo).

Abstract
Khan, F. K. 2025. The Origin and Diversification of Ectomycorrhizal Fungi. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2534. 56 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-2470-8.

Ectomycorrhiza (ECM) is a symbiotic relationship between fungi and plants, which is essential for many woody plants. ECM fungi have evolved independently multiple times in several lineages from saprotrophic ancestors. However, the origin and diversification processes of ECM lineages are still not well understood. In Paper I, we addressed whether ECM lineages evolved independently or if reversals to saprotrophy occurred. These hypotheses were tested using model-based methods and a phylogeny based on 2 174 ECM taxa. We concluded that our inferences about ECM evolution are affected by whether a rate shift in different time periods or ECM clades is allowed, and that reversals to saprotrophy are probably rare. In Paper II, I investigated whether the ECM-linked genomic changes occurred abruptly at the transition to the ECM lifestyle or were part of a trend before or after the transition in ECM lineages. I compared 26 genomes from ECM species in Inocybaceae with six saprotrophic outgroups. The findings suggest that the molecular changes, important for transition to the ECM lifestyle, occur in proximity to the origin of this lifestyle in Inocybaceae. In Paper III, I explored whether genomic diversity in ECM lineages is shaped by independent transitions or due to diversification within each lineage. I compiled a phylogenetically diverse dataset of 75 genomes, representing eight ECM lineages. The findings show that while genomic diversity from lineage-level additions quickly saturates for all enzyme groups investigated, species-level additions continue to contribute new diversity beyond the limits of our dataset. In Paper IV, we tested the mutual exclusivity of psilocybin and muscarine in Inocybaceae species by screening 21 samples. I also explored the origin and evolution of genes involved in psilocybin production in these species using 24 genomes. We detected both psilocybin and muscarine, for the first time, in a single species of Inocybaceae and also concluded that the psilocybin gene cluster has evolved through convergent evolution, with two possible origins of the gene cluster within the family. Overall, this thesis contributes to improving our understanding of ECM fungal evolution and highlights how traits that appear alike can emerge through distinct evolutionary processes.

Keywords: Symbiosis, Ectomycorrhiza, Evolution, Saprotrophy, Phylogeny, Comparative Genomics

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ISSN 1651-6214
ISBN 978-91-513-2470-8
URN urn:nbn:se:uu:diva-552928 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-552928)

To my family

List of Papers

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

I. Sanea Sheikh, **Faheema Kalsoom Khan**, Mohammad Bahram and Martin Ryberg. 2022. Impact of model assumptions on the inference of the evolution of ectomycorrhizal symbiosis in fungi. *Scientific Reports*, 12 (1), 1-10. doi: https://doi.org/10.1038/s41598-022-26514-2

II. **Faheema Kalsoom Khan**, Marisol Sanchez-Garcia, Hanna Johannesson and Martin Ryberg. 2024. High rate of gene family evolution in proximity to the origin of ectomycorrhizal symbiosis in Inocybaceae. *New Phytologist*, 244(1). doi: https://doi.org/10.1111/nph.20007

III. **Faheema Kalsoom Khan,** Marisol Sanchez-Garcia and Martin Ryberg. 2025. Species or lineages: What matters more for the functional diversity of ectomycorrhizal fungi? *Manuscript*.

IV. **Faheema Kalsoom Khan**, Markus Hiltunen, Taj Muhammad, Alexander Bradshaw, Ali Awan, Ellen Larsson, Bryn Dentinger, Ulf Göransson, Hanna Johannesson and Martin Ryberg. 2025. Complex evolutionary history of the psilocybin production pathway in Inocybaceae. *Manuscript*.

Additional Papers

The following papers were published or submitted during the course of my PhD studies but are not part of this thesis:

1. Gregory Bonito, Shingo Miyauchi, Richard D. Hayes, Hao Tan, Alan Kuo, Aaron Robinson, Xinxin Wang, Jud Van Wyk, Annegret Kohler, Laure Fauchery, Elodie Drula, Gian Maria Niccolo Benucci, Emmanuelle Morin, Martin Ryberg, **Faheema Kalsoom Khan**, Melissa Cravero, Reid Longley, Kerrie Barry, Alicia Clum, Kecia Duffy, Anna Lipzen, Kurt LaButti, Robert Riley, Vasanth Singan, Andrei Steindorff, Jie Wang, Tom Bruns, Sundy Maurice, Philippe Clowez, Kerry O'Donnell, Saskia Bindschedler, Pilar Junier, Patrick Chain, Igor V Grigoriev, Francis M. Martin. Pangeneric analyses reveal the divergent genome evolution and ecologies between morels and truffles in the Morchellaceae. 2025. *Nature Communications (Submitted).*
2. R. Henrik Nilsson, Arnold Tobias Jansson, Christian Wurzbacher, Sten Anslan, Pauline Belford, Natàlia Corcoll, Alexandra Dombrowski, Masoomeh Ghobad-Nejhad, Mikael Gustavsson, Daniela Gómez Martinez, **Faheema Kalsoom Khan**, Maryia Khomich, Charlotte Lennartsdotter, David Lund, Breyten Van Der Merwe, Vladimir Mikryukov, Marko Peterson, Teresita M. Porter, Sergei Palme, Alice Retter, Marisol Sanchez-Garcia, Sten Svantesson, Patrik Svedberg, Duong Vu, Martin Ryberg, Kessy Abarenkov, Erik Kristiansson. 20 years of bibliometric data illustrates a lack of concordance between journal impact factor and fungal species discovery in systematic mycology. 2024. *Mycokeys*. 110: 273–285.
3. Anna Rosling, Shadi Eshghi Sahraei, **Faheema Kalsoom Khan**, Alessandro Desirò, Abigail Bryson, Stephen Mondo, Igor Grigoriev, Gregory Bonito and Marisol Sánchez-García. 2024. Evolutionary history of arbuscular mycorrhizal fungi and genomic signatures of obligate symbiosis. *BMC genomics*, 25(529).
4. Martin Ryberg, **Faheema Kalsoom Khan** and Marisol Sánchez García. 2022. On the evolution of ectomycorrhizal fungi. *Mycosphere*, 13 (2).

5. Sylvestre A. Badou, Brendan Furneaux, André De Kesel, **Faheema Kalsoom Khan**, Roel D. Houdanon, Martin Ryberg and Nourou S. Yorou. 2022. *Pseudopaxillus africanus*, gen. et sp. nov., a novel lamellate bolete from West Africa. Fungal Resources. *Mycological Progress*, 21: 243–256.
6. Marisol Sánchez-García, Martin Ryberg, **Faheema Kalsoom Khan**, Torda Varga, László G. Nagy and David S. Hibbett. 2020. Macroevolutionary dynamics of fruiting body forms and nutritional modes in mushroom-forming fungi based on an 8400 species phylogeny. *PNAS,* 117 (51): 32528-32534.
7. **Faheema Kalsoom Khan***, Kerri Kluting*, Jeanette Tångrot, Hector Urbina, Tea Ammunet, Shadi Eshghi Sahraei, Martin Rydén, Martin Ryberg and Anna Rosling. 2020. Naming the untouchable – environmental sequences and niche partitioning as taxonomical evidence in fungi. *IMA Fungus*, 11, 23.
8. Venkat Talla**, Faheema Kalsoom Khan**, Daria Shipilina, Irina Marova and Niclas Backström. 2017. Heterogeneous patterns of genetic diversity and differentiation in European and Siberian chiffchaff (*Phylloscopus collybita abietinus/P. tristis*). *G3*, 7(12): 3983-3998.
9. Venkat Talla, Alexander Suh, **Faheema Kalsoom Khan**, Vlad Dinca, Roger Vila, Magne Friberg, Christer Wiklund and Niclas Backström. 2017. Rapid increase in genome size as a consequence of transposable element hyperactivity in wood-white (*Leptidea*) butterflies. *Genome Biology and Evolution*, 9(10): 2491-2505.

* Equal contributions

Contents

Abbreviations

ECM	Ectomycorrhiza
AM	Arbuscular Mycorrhiza
PCWDEs	Plant Cell Wall Degrading Enzymes
CAZymes	Carbohydrate Active enZymes
MiSSPs	Mycorrhiza induced Small Secreted Proteins
TEs	Transposable Elements
LSU	Large Sub Unit
SSU	Small Sub Unit
RPB	RNA Polymerase B
SM	Secondary Metabolites
AIC	Akaike Information Criterion
GH	Glycoside Hydrolases
GT	Glycosyl Transferases
CE	Carbohydrate Esterases
PL	Polysaccharide Lyases
CBM	Carbohydrate Binding Modules
AA	Auxiliary Activities
SSP	Small Secreted Proteins
WGS	Whole Genome Sequencing
SMs	Secondary Metabolites
BLAST	Basic Local Alignment Search Tool
KAT	Kmer Analysis Toolkit
BUSCO	Benchmarking Universal Single-Copy-Orthologs

Disclosure statement on the use of Artificial Intelligence tools

"*This thesis was written by the author without content generation using text generative artificial intelligence (AI) tools. Where AI was used, it was in line with the Department of Organismal Biology's policy, such as for grammar and style refinement, translation, or coding assistance, with full oversight and verification by the author.*"

Introduction

"From so simple a beginning, endless forms most beautiful and most wonderful have been, and are being, evolved."

Charles Darwin

One of the fascinating features of the living world is its immense diversity of organisms. The kingdom Fungi constitutes one of the most diverse group of organisms in terms of forms, functions, sizes, habitats and lifestyles. They are ubiquitous and can exist in a range of forms, from simple microscopic to large and complex fruiting bodies like mushrooms. The versatile lifestyles exhibited by fungi include saprotrophy and symbiosis.

As evolutionary and systematic biologists, we strive to document and explain the diversity of organisms. Ectomycorrhizal fungi (ECM) are one of the highly diverse group of symbiotic fungi, with more than 20 000 species in upto 87 clades (Rinaldi, Comandini and Kuyper, 2008; Tedersoo, May and Smith, 2010; Tedersoo and Smith, 2017). The overarching aim of my thesis is to understand the origin and the evolutionary processes that drive the diversity of ECM fungi. In different chapters of this thesis, I investigate key questions about their evolution using phylogenetic and genomic approaches.

In the following sections, I will first give a brief introduction to the ECM fungi and explain the hypotheses regarding their origins. I will also touch upon the fungal secretome and its relevance to the genome evolution in ECM species. I will then present a brief overview of the methods used in this thesis, such as how DNA from these symbiotic fungi was obtained and sequenced, as well as the specific pipelines used to analyse the data.

Trophic modes in Fungi

Fungi are heterotrophic and the two major trophic modes in fungi are saprotrophy and symbiotrophy, the latter of which includes mycorrhizal associations as a major part. Saprotrophic fungi get their nutrition by decomposing dead organic matter whereas mycorrhizal fungi fulfil their nutritional demands by associating with living plants. These fungi have a profound impact on the global ecosystem in both of their ecological roles as decomposers or

mycorrhizal associates (Baldrian *et al.*, 2012; Tedersoo, Bahram and Zobel, 2020). The decomposition activities performed by saprotrophic fungi facilitate the release and subsequent availability of nutrients for uptake by other organisms, as well as the recycling of carbon into the atmosphere (Baldrian *et al.*, 2012). Different plants, including crops and forest trees, rely on mycorrhizal fungi for the acquisition of essential mineral nutrients like nitrogen, phosphorus etc. Fungi, thus, play the dual roles of providing nutrients to plants and releasing nutrients back into ecosystems.

Saprotrophy

Saprotrophic fungi generally degrade diverse sources of organic materials from animals, plants and other organisms. However, the saprotrophic fungi that decompose plant materials in terrestrial forest ecosystems are of particular relevance to my research in this thesis.

Different varieties of plant-degrading saprotrophic fungi vary in their capacity to break down plant cell walls. Among them, white-rot fungi are the most aggressive in terms of plant decomposition. They can break down all major polysaccharide structural components of plant cell walls, including cellulose, hemicellulose, and lignin (a highly recalcitrant and complex polymer), leaving decayed wood with a white-coloured, fibrous, rotting texture. Hence, they are termed as white-rot fungi. On the other hand, brown-rot fungi degrade cellulose and hemicellulose in plant cell walls but do not significantly degrade lignin, leaving behind brown coloured wood remains after selectively decomposing cell wall components. Soft rots, however, prefer wood with less lignin and higher moisture content, producing characteristic cavities in the rotting wood (Daniel and Nilsson, 1997). Similar to white-rot fungi, soil/litter decaying fungi break down organic matter in the litter layer, contributing to the formation of the so-called white humus (Osono, 2006). Some species in this group have the ability to break down lignin, though less effectively than wood rotting white-rot fungi (Floudas *et al.*, 2020).

Mycorrhiza

Mycorrhiza is the most common symbiotrophic lifestyle in fungi. The word “Myco” means “fungus” and “Rhiza” means “root”. So, mycorrhiza literally means “fungus roots” and represents a symbiotic relationship between fungal hyphae and plant roots, where fungi trade nutrients like nitrogen and phosphorus as well as water, for photosynthetically fixed sugars from plants. In this relationship, fungi can also provide their symbiotic partners with protection from a range of stressful conditions, such as drought, parasite load, and chemical toxicity etc. Mycorrhizal associations exist in more than 90% of plants, and are crucial for their survival (Smith and Read, 2008). This mutualistic relationship between fungi and plants is ancient, and mycorrhizal fungi are

hypothesized to have assisted early land plants colonize the terrestrial environment. Several lines of evidence, including the direct evidence of the earliest fossil records showing mycorrhiza-like associations, support this hypothesis (Pirozynski and Malloch, 1975). There are different types of mycorrhizae, with Arbuscular Mycorrhiza (AM) and ectomycorrhiza (ECM) being the most common types.

Ectomycorrhiza

ECM fungi are one of the most diverse types of mycorrhizal fungi, including over 20 000 species (Rinaldi, Comandini and Kuyper, 2008). This symbiosis is distinguished from other types of mycorrhizae by specific morphological (symbiotic) structures, such as the Hartig net and mantle (Fig. 1). The Hartig net, an intercellular network of fungal hyphae inside plant roots, is the primary site of nutrient exchange between fungi and their host plants, while the mantle is a thin layer of fungal hyphae that covers plant roots and stores nutrients. In contrast to the other widespread type of mycorrhiza, AM, fungal hyphae in the ECM relationship do not penetrate plant cell walls.

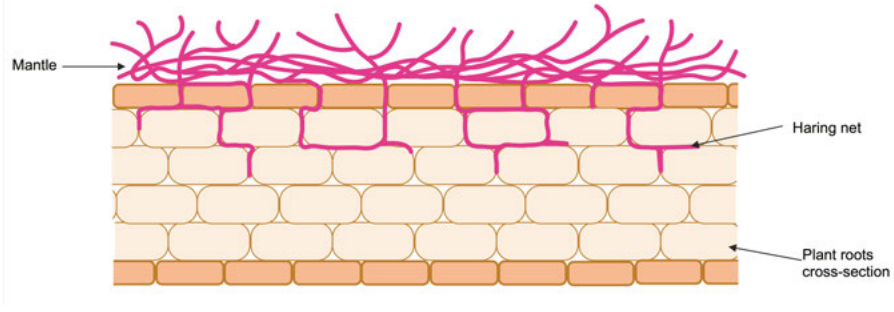

Figure 1. A schematic illustration of ECM fungal symbiosis with plant roots, showing the formation of a mantle and a Hartig net.

The majority of fungi involved in ECM associations are macrofungi, which include highly prized edible mushrooms such as truffles, chanterelles, matsutake, and porcini. Large woody timber-producing trees of economic importance, such as pine, spruce, oak, beech and birch, are among the plant partners of ECM fungi. Although only 2% of land plants form ECM associations, these plants represent most of the dominant tree species and account for 60% of tree stems globally (Steidinger *et al.*, 2019).

The different fungal lineages that form ECM belong to the sub-phyla of fungi i.e., Mucoromycotina, Pezizomycotina, and Agaricomycotina (Tedersoo, May and Smith, 2010; Yamamoto *et al.*, 2017). Only Endogonaceae in Mucoromycotina contains ECM lineages, and Pezizomycotina and Agaricomycotina contain the majority of ECM lineages. There are ca. 82-87 lineages of ECM in total

(Tedersoo, May and Smith, 2010; Tedersoo and Smith, 2013, 2017), of which three lineages belong to Mucoromycotina, ca. 32 lineages belong to Pezizomycotina and 46 lineages to Agaricomycotina (Tedersoo, May and Smith, 2010).

All ECM lineages have non-ECM sister lineages, most of which have saprotrophic ecology (Tedersoo and Smith, 2013). Inocybaceae is one of the diverse, mushroom-forming lineages of ECM in Agaricales, with over 1 050 estimated species (Matheny *et al.*, 2006). It forms mycorrhizal symbioses with 23 families of vascular plants and has a worldwide distribution (Matheny and Kudzma, 2019). This lineage is also known for the toxicity of its species, as some species in this family produce different toxins including hallucinogenic psilocybin and neurotoxic muscarine (Stijve, Klan and Kuyper, 1985). Its relationship with the sister lineage Crepidotaceae, which has saprotrophic ecology, is well established (Fig. 2). Inocybaceae lineage is the focus of my research in **Paper II** and **Paper IV**.

Figure 2. (a) *Inocybe sp.* and (b) *Crepidotus caspari*. These species are examples of ECM and saprotrophic lifestyles in fungi, respectively, and also represent the lineages of focus in the comparative genomic studies in **Paper II** and **Paper IV** (Inocybaceae only). Photos (a) by Martin Ryberg and (b) by Petra Korall.

Origin of ECM fungi

Previous research has suggested that different ECM lineages evolved independently multiple times from ecologically diverse saprotrophs, such as brown-rot, white-rot, litter and soil/litter decaying fungi (Kohler *et al.*, 2015). However, it is still unclear precisely how the ECM lineages diverged from their saprotrophic precursors, and the question of how ECM originated remains open.

There are numerous theories that attempt to explain the origin and overall diversification processes of different ECM lineages. A highly debated theory regarding the origin of ECM fungi states that once the ECM lifestyle was acquired, there were several reversals back to the saprotrophic habit, suggesting

instability of this lifestyle (Hibbett, Gilbert and Donoghue, 2000). Another common and widely accepted view on ECM fungal evolution is that there were 82-87 independent origins of ECM lineages, with no reversals to saprotrophy (Bruns and Shefferson, 2004; Tedersoo, May and Smith, 2010; Tedersoo and Smith, 2017). In these multiple independent transitions, each ECM lineage is counted as a separate origin of this lifestyle.

To explain the diversity of ECM fungi, some hypotheses posit that different geological time periods were important for the evolution of these lineages. Specifically, the timing of ECM host plant origins is frequently correlated with the origin of ECM symbiosis and are, thus, suggested to be key to the diversification of different ECM lineages e.g the origin of plants in Pinaceae in the Late Jurassic and of different angiosperm hosts from the Betulaceae and Fagaceae families in the Early Cretaceous period (Halling, 2001). Another hypothesis in this regard is that the ECM lifestyle is a key innovation that led to adaptive radiations of ECM lineages after the transition to ECM due to the availability of new niche spaces and adaptive radiations imply increased diversification rates in ECM lineages. However, this viewpoint is not widely supported, as studies have shown increased diversification rates only in particular lineages in correlation with the shift to the ECM lifestyle (Ryberg and Matheny, 2012; Sánchez-García and Matheny, 2017).

Resolving the conflicts regarding the origin of ECM is necessary for properly interpreting how functional traits, such as gene copy numbers of carbohydrate degrading enzymes in ECM fungi, have evolved. Very few studies have analytically tested the hypotheses related to ECM origin using inclusive datasets that cover all ECM lineages and appropriate models (Sánchez-García *et al.*, 2020). Since higher rates of evolution have been hypothesized for different geological time periods and lineages in ECM, these hypotheses also need to be tested using models that allow rate shifts through time and across clades, together with a comprehensive ECM dataset. The hypotheses of whether there were reversals to saprotrophy or multiple independent origins of ECM, and whether different time periods were important for the evolution of ECM lineages are tested in **Paper I**.

Fungal Secretome

Before delving into the genomics of ECM fungi, it is important to understand the fungal secretome, its components and significance generally for all fungi and specifically for ECM and saprotrophic fungi. I will briefly walk you through this in the following section.

The “secretome” as a term was coined in 2000 and initially described the complete secretory machineries in bacteria (Tjalsma *et al.*, 2000). The modern definition of secretome is broader and encompasses other organisms as well (Krause *et al.*, 2013). The fungal secretome, in particular, refers to the full set

of proteins and other molecules that are synthesized within fungal cells and either fully released into the extracellular environment or remain attached to the outer cell walls (Girard *et al.*, 2013). As exodigesters, fungi depend on these secreted proteins to degrade complex biopolymers in order to absorb nutrients, construct and modify their cell walls, communicate through signalling, and compete with other microbes (Filiatrault-Chastel *et al.*, 2021). The secretome is, therefore, essential for the overall survival and development of fungi as well as for their interactions with hosts and environment. Moreover, fungi can alter their secretome to adapt to environmental shifts such as changes in carbon and nitrogen sources (Brown, Ries and Goldman, 2014). Thus, the diverse array of enzymes, tailored to their specific lifestyles and habitats, enable them to thrive in various ecological niches (McCotter, Horianopoulos and Kronstad, 2016; Hage and Rosso, 2021). The evolution of different components of the fungal secretome in ECM fungi is explored in **Paper II** (CAZymes, peptidases, lipases, including their intracellular counterparts, small secreted proteins and secondary metabolites), **Paper III** (CAZymes, peptidases, lipases) and **Paper IV** (secondary metabolite like psilocybin). I will briefly introduce each in the following section.

CAZymes

Carbohydrate Active enZymes (CAZymes) are a key component of fungal secretome, which are involved in the deconstruction, modification and formation of complex polysaccharides (Lombard *et al.*, 2014). An important subset of CAZymes, relevant in the context of this thesis, are Plant Cell Wall Degrading Enzymes (PCWDEs). These enzymes break down different structural components of plant cell walls such as cellulose, hemicellulose, lignin and pectin (Chang *et al.*, 2016). CAZymes, based on the protein sequence and structure similarities, are further divided into different classes, i.e glycoside hydrolases (GH), glycocyltransferases (GT), carbohydrate esterases (CE), polysaccharide lyases (PL), carbohydrate binding modules (CBM) and auxiliary activities (AA) (Cantarel *et al.*, 2009). Cellulose generally forms tightly packed and highly crystalline microfibrils, making it difficult to degrade (Klemm et al., 2005). To deconstruct cellulose, fungi utilize specialized CAZymes like cellulases (endoglucanase, β-glucosidase and cellobiohydrolase) (Battaglia *et al.*, 2011). These enzymes belong to the GH class, with GH6 and GH7 particularly employed for this role (Zerillo *et al.*, 2013). Hemicellulose is targeted by xylanases, chitinases, and endo-β-1,3-glucanases, also from the GH class, and xylan esterases from the CE class, among others (Broeker *et al.*, 2018; Sista Kameshwar and Qin, 2018; Kameshwar, Ramos and Qin, 2019). Lignin, is primarily degraded by lignin modifying enzymes such as laccases and peroxidases from the AA class (Zhang *et al.*, 2021). In addition, lytic polysaccharide monooxygenases (LPMOs) from the AA class contribute to the

breakdown of cellulose, chitin and starch (Borisova *et al.*, 2015). The CAZymes acting on pectin include pectin lyases from the PL class (Yadav *et al.*, 2009).

Saprotrophic fungi, as free-living organisms, rely on a broad range of CAZymes to acquire nutrients. White-rot and soil/litter decaying fungi share a similar enzymatic apparatus for cellulose breakdown but differ in the degradation of hemicellulose and lignin. In contrast, brown-rot fungi degrade cellulose, and to a small extent lignin, using hydroxyl radicals generated by the Fenton reaction (Floudas *et al*., 2020).

Peptidases

Peptidases are another major component of the fungal secretome. These enzymes cleave peptide bonds, digesting larger proteins into smaller peptides and amino acids (Rawlings *et al.*, 2014). Peptidases are classified on the basis of the amino acid partaking in the hydrolysis process. For example, if the active site contains serine, they are classified as serine peptidases; if it involves aspartic acid, they are aspartic peptidases etc (Barrett and Rawlings, 1995). In addition to their role in nutrient acquisition in fungi, peptidases also have roles in other functions such as cell signalling, and suppression of the host immune system, for instance, in symbiotic fungi (Xia, 2004; van der Hoorn, 2008).

Lipases

Lipases are the third largest category of digestive enzymes after CAZymes and peptidases (Kumar *et al.*, 2023). These enzymes hydrolyse triglycerides to glycerol and fatty acids, facilitating the intake, transport and processing of lipids in most organisms (Salah *et al.*, 2006). Lipases have been broadly divided into three main classes i.e GX, GGGX and Y in the Lipase Engineering Database according to the amino acid in their oxyanion hole (Fischer and Pleiss, 2003). Fungal lipases are mostly extracellular (Geoffry and Achur, 2018) and highly substrate specific (Gopinath *et al.*, 2013; Kumar *et al.*, 2023). The additional roles of lipases in biological processes include synthesis of membrane lipids (Zanghellini *et al.*, 2008) and symbiosis (Kameoka and Gutjahr, 2022).

Small Secreted Proteins

Small Secreted Proteins (SSPs) are small signalling proteins, typically less than 300 amino acids in length, and contain a signal peptide (Feldman, Yarden and Hadar, 2020). Most of these proteins are encoded by genes, with unknown functions and very little homology to reference sequences in the databases. Since only a few of these genes are conserved at the sequence level, they are hence called orphan genes (Feldman *et al.*, 2017). Some studies suggest that

these proteins could be more structurally conserved, which is less apparent from their primary sequences (Lo Presti *et al.*, 2015; Selin *et al.*, 2016). The SSPs constitute ca. 40-60% of the fungal secretome (Pellegrin *et al.*, 2015). SSPs, studied extensively in fungi, are characterized by their cysteine-rich composition, and have been shown to have a role in fungal-host interactions in both pathogenic and mutualistic fungi (Veneault-Fourrey and Martin, 2011; Lo Presti *et al.*, 2015). Given their ability to suppress host immune system, they are often referred to as effectors-like proteins (Alfaro *et al.*, 2014; Rovenich, Boshoven and Thomma, 2014; Lo Presti *et al.*, 2015). It has been proposed that their rapid divergent evolution may result from strong selection pressure as many genes involved in host-fungal interactions are constantly subjected to such pressure (Aguileta *et al.*, 2009; Plissonneau *et al.*, 2017). In ECM fungi, a handful of SSPs have been functionally characterized (Pellegrin *et al.*, 2015). The sub-category of SSPs i.e Mycorrhiza-induced Small Secreted proteins (MiSSPs) like MiSSP7 and MiSSP8, have been found to be up-regulated in the initial stages of mycorrhiza formation in *laccaria bicolor* and MiSSP7 has been found to have a role in dampening the host immune responses, and these mycorrhiza specific SSPs are thus suggested to be key for the ECM symbiosis (Plett and Martin, 2015; Pellegrin *et al.*, 2019).

Secondary metabolites

Secondary metabolites (SMs) can be defined as the chemical compounds that are not required for an organism's growth, development and reproduction, and serve various other functions for the producing organisms such as providing defence against other organisms, facilitating interactions with their environments and supporting reproductive processes (Vaishnav and Demain, 2011; Avalos and Limón, 2021). These compounds are produced by many organisms including plants, animals, and microbes such as bacteria and filamentous fungi (Conrado *et al.*, 2022). Examples of SMs commonly found in fungi are melanin, penicillin, and lovastatin etc. The major classes of fungal SMs based on their chemical structure are polyketides (PKS: polyketide synthases), non-ribosomal peptides (NRPS: non-ribosomal peptide synthetases), hybrid polyketides/non-ribosomal peptides (PKS/NRPS) and terpenoids (TC: terpene cyclases) (Avalos and Limón, 2021). Genes involved in secondary metabolism pathways are typically arranged in clusters within the genome (Medema *et al.*, 2015; García-Estrada *et al.*, 2018). These clusters usually contain a gene for the main signature enzyme (such as polyketide synthase) that produces the backbone structure of the compound. In addition, there are genes for tailoring enzymes (such as methyltransferases, oxidoreductases and kinases etc) which further modify and refine the structure (Osbourn, 2010; Keller, 2019). Transporters, regulatory genes and genes of unknown function are sometimes also located within the same gene cluster (de Almeida, Taulk-Tornisielo and Carmona, 2013; Reynolds *et al.*, 2018). It has been proposed that physical

proximity of these genes allows, their coordinated regulation and expression at the chromatin level, and, also their coinheritance by horizontal gene transfer or vertically from one generation to another (Osbourn, 2010; Reyes-Dominguez *et al.*, 2010).

Psilocybin is an example of a fungal SM, which is of great importance due to its pharmaceutical applications (Johnson and Griffiths, 2017; Johnson *et al.*, 2019; Meyer and Slot, 2023). It is produced by multiple genera of psychedelic mushrooms, including *Inocybe* in Inocybaceae (Wurst, Kysilka and Flieger, 2002; Dinis-Oliveira, 2017). Previous research has identified a distinct gene cluster for psilocybin synthesis in an Inocybaceae species, likely resulting from convergent evolution. However, the origin and evolution of the genes involved in psilocybin production within Inocybaceae remain unknown. The evolutionary dynamics of these genes in Inocybaceae are the focus of investigation in **Paper IV**.

Genomics of ECM fungi

The first genome of an ECM fungus i.e *Laccaria bicolor* was published in 2008 (Martin *et al.*, 2008), followed by the genome of *Tuber melanosporum* (Martin *et al.*, 2010). The main features of these genomes were a low number of genes encoding CAZymes, particularly PCWDEs, a high proportion of transposable elements (TEs) compared to other fungal genomes sequenced at that time, and the upregulation of MiSSPs in ECM root tips. To confirm whether these patterns are general in ECM fungal genomes, the first large scale comparative genomic study consisting of 13 ECM fungal genomes was carried out by Kohler *et al.* (2015). This study highlighted a number of ECM-related genomic changes compared to their closely related saprotrophic relatives. These molecular changes linked to the ECM symbiosis were termed as molecular signatures of ECM and were also referred to as the so-called genomic toolbox required for the development of symbiosis in ECM fungi.

The most striking genomic changes observed in ECM fungi were the contractions of genes encoding CAZymes, involved in lignin and cellulose degradation, and the turnover of symbiosis-specific SSPs when compared to their saprotrophic relatives. These findings suggest that ECM fungi have lost many ancestral carbohydrate-degrading proteins, which are no longer needed in their symbiotic lifestyle, and have also acquired new genes required for the transition to this new lifestyle of ECM. The contraction of CAZyme encoding genes has since been reported in several other ECM lineages and remains one of the most prevalent molecular signatures in ECM fungal genomes (Peter *et al.*, 2016; Murat *et al.*, 2018; Hess *et al.*, 2018; Miyauchi *et al.*, 2020; Wu *et al.*, 2022; Looney *et al.*, 2022). The later studies showed that ECM fungi from other lineages often contain moderate to low content of SSPs in their genomes (Hess *et al.*, 2018; Murat *et al.*, 2018; Looney *et al.*, 2022).

The main highlight of an ECM genomic study by Peter *et al.* (2016), published shortly after Kohler *et al.* (2015), was that the ECM lineages have significantly larger genomes than their saprotrophic relatives due to TEs proliferation. The physical genomic proximity of TEs to SSPs was later shown by other studies (Looney *et al.*, 2022; Wu *et al.*, 2022). These studies suggest that TE activities contribute to genome expansions in ECM species and play a role in the duplications of SSPs.

Hess *et al.* (2018) found that the previously observed ECM-related genomic changes were already encoded in the genomes of ancestral saprotrophs and, that these orthologous ancestral genes were co-opted by ECM species to serve new ECM-related functions (Hess *et al*., 2018). Miyauchi *et al.* (2020) highlighted the divergent evolution and diversification of lineage-specific novel genes in ECM lineages. The expansion of such lineage-specific genes, unique to each ECM lineage, has also been observed across different ECM lineages in multiple genomic studies. For example, Suillineae genomes were characterized by expansions of SM gene clusters linked to host specificity (Lofgren *et al.*, 2021), *Cenococcum geophilum* genome by the upregulation of water channel and sugar transporter genes (Peter *et al.*, 2016) and Tuberaceae genomes by the expansions of genes related to the fruiting-body development (Murat *et al.*, 2018). Other general genomic changes associated with ECM species include a smaller number of SM gene clusters as well as fewer peptidases (serine peptidases) and lipases (versatile lipases) in the genomes of ECM species, compared to their free-living saprotrophic relatives (Pellegrin *et al.*, 2015; Muszewska *et al.*, 2017; Lebreton *et al.*, 2021).

Large-scale genomic studies and the rapidly expanding number of genomes sequenced from different ECM fungal lineages have begun to scratch the surface of the molecular underpinnings of ECM symbiosis. However, we are still far from getting a complete picture of the molecular machinery required for this symbiosis (Lebreton *et al.*, 2021). There is a need for more genomes from both diverse ECM lineages and additional representatives within individual lineages to identify other key genes involved in the establishment and regulation of this symbiosis, as well as to pinpoint the evolutionary events that mediated the transition to this lifestyle. These questions are the focus of **Paper II**. Furthermore, as more ECM fungal genomes from different lineages are becoming available, new opportunities arise to gain a comparative perspective on genomic and functional diversity, both within and between lineages, by exploring how different species can contribute to soil enzymatic activity through their secretome. This aspect is the focus of **Paper III**.

Obtaining and sequencing DNA from the ECM fungal symbionts

In the section below, I will briefly explain the main methods used in this thesis, starting with DNA extractions, followed by sequencing, and then data analyses. The details can be found in the respective papers.

The conventional method to obtain DNA from fungi is through culturing, where mycelium or other cells are grown and propagated under controlled laboratory conditions (White *et al.*, 1990). This technique is particularly useful when the goal is to extract DNA from a single fungal species, since pure samples cannot be obtained directly from nature (Wijayawardene *et al.*, 2021). Cultures are typically free from other contaminating organisms, if maintained properly (Jong and Birmingham, 2001). DNA extracted from cultures is generally of high quality and quantity, which is often required for various downstream applications including different genome sequencing techniques (Robin *et al.*, 2016; Bellemare, John and Marqueteau, 2018). However, many host-associated fungi are unculturable under laboratory conditions (Rahimlou, Quandt and James, 2024). Cultivating ECM fungi in laboratory settings is particularly challenging. While some ECM species can be grown in culture, their growth rates vary, with some growing slowly and others relatively faster in the laboratory. Nevertheless, successful cultivation is not always possible without the presence of their host plants, both in the laboratory and in nature (Douhan *et al.*, 2011).

Metagenomic approaches offer culture-independent alternatives for studying the genomes of unculturable organisms. In these methods, the genomic DNA is extracted directly from the environmental samples, which circumvents the need to culture the study organisms (Schloss and Handelsman, 2005). Given the small genome sizes of fungi, near-complete genomes can be obtained using DNA directly from the fungal sporocarps (fruiting bodies) (Bahram *et al.*, 2018). The fresh sporocarps in the field can be preserved by either freezing in liquid nitrogen (Chang *et al.*, 2019; Looney *et al.*, 2022; Wu *et al.*, 2022) or dried by desiccation, for example using food dehydrator (Bahram *et al.*, 2018; Khan *et al.*, 2024). The later method facilitates filed-work by making the transport and storage of samples easier, allows sampling of many specimens, and also makes DNA extraction from old specimens possible (Khan *et al.*, 2024). This technique has been used in **Paper II**, **III**, and **IV**. However, when studying secondary metabolites and their associated gene clusters, fresh samples should preferably be used as some secondary metabolites, such as tryptamine alkaloids, have poor stability (Stijve, Klan and Kuyper, 1985; Gotvaldová *et al.*, 2021) which can pose challenges for their detection. Also, dried samples may yield fragmented DNA, which can be problematic for the retrieval of complete gene clusters from the genomes. Additionally, dried samples are not ideal for transcriptomics studies.

Whole genome sequencing

After DNA extraction, the next major step in genomics is genome sequencing. In this thesis, whole genome sequencing (WGS) was used to sequence all fungal genomes. In WGS, which is a type of shotgun sequencing, an organism's entire genome is sheared into thousands of small fragments, each fragment is

sequenced individually and the resulting sequences are then assembled bioinformatically to reconstruct the complete genome (Weber and Myers, 1997). It is one of the most popular applications of high throughput sequencing technologies such as Illumina, PacBio (Pacific Biosciences) and Oxford Nanopore (Park and Kim, 2016).

These sequencing platforms differ in terms of read length, error rates, throughput and cost (De Maio *et al.*, 2019). The quality and quantity of the input DNA can also vary depending on the chosen technology (Murigneux *et al.*, 2020). Therefore, these parameters and the research questions at hand must be carefully considered when selecting a sequencing technology.

Illumina sequencing provides a good yield with a low error rate, making it suitable for studying e.g gene family evolution and phylogenomic relationships between different organisms. In this thesis, Illumina sequencing was used to sequence whole genomes of different ECM fungi in **Paper II** and **Paper III**, where the focus was broadly on the study of gene family evolution in multiple ECM species and lineages.

PacBio sequencing with its long-read capabilities, is ideal for analysing complex regions of the genomes such as repetitive sequences and gene synteny. Due to its relatively high cost, it is particularly advantageous when working with a limited number of samples (De Maio et al., 2019; Murigneux et al., 2020). In **Paper IV**, PacBio sequencing was used to sequence the genomes of two psilocybin-producing Inocybaceae species as the study focused on investigating the evolution of psilocybin gene clusters.

Filtration of contaminant reads from sequencing data

While using sporocarp tissues to generate genomic DNA from unculturable fungi is a promising approach, it comes with its own set of challenges. For instance, sporocarps can host a diverse range of organisms, including bacteria, fungicolous fungi, and insect larvae such as those of flies and beetles (Schigel, 2011; Maurice *et al.*, 2021; Lunde *et al.*, 2023). Sequencing using sporocarp tissues will also capture the genomes of the associated organisms (Khan *et al.*, 2024). Additionally, the presence of contaminants in the assemblies can lead to inflated BUSCO scores (**Paper II**). Therefore, it is important to bioinformatically filter out reads sourcing from non-target organisms to ensure that only the desired genome is analysed.

This filtration process can be performed at the read level, the assembly level, or both. I employed it at both levels in this thesis. Contaminants at the read level were identified based on low coverage and deviating GC content of the k-mers, using k-mer spectra in the Kmer Analysis Toolkit (KAT) (Mapleson *et al.*, 2017). Contaminants at the assembly level were identified by the BLAST hits of contigs against the GenBank nucleotide (nt) database, and by analysing the Blobplots (two-dimensional scatter plots with GC and coverage histograms) generated using BlobTools (Laetsch and Blaxter, 2017).

The identified bacterial reads comprising the contaminated contigs were removed from assemblies using workflow A in BlobTools. The remaining reads were then re-assembled and re-checked for possible bacterial contaminations. This process was repeated iteratively until no contigs with bacterial contamination were identified (Fig. 3).

Contigs derived from other fungal contaminants, such as Ascomycetes, were identified on the basis of their low coverage and confirmed via BLAST searches of these contigs against the GenBank nucleotide (nt) database (Altschul *et al.*, 1990). The reads comprising these contaminant contigs were then removed from the total read set. Their removal was verified by BLASTing the re-assembled assemblies against a custom database containing ribosomal large subunit sequences from both common contaminants and the target species. This pipeline for contaminant read filtering was used in **Papers II, III** and **IV**.

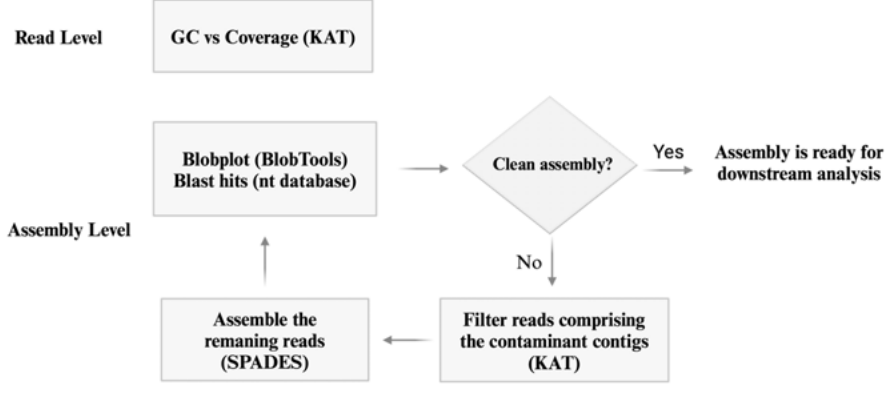

Figure 3. Pipeline used to filter reads sourcing from contaminants in this thesis

Comparative Genomics/Phylogenomics

The main approach used for data analyses in this thesis was phylogenomics, where the evolutionary relationships of organisms are studied by comparative analyses of whole genomes or substantial proportions of them, rather than using a few genes (O'Brien *et al.*, 1999; Delsuc, Brinkmann and Philippe, 2005). The general workflow (Fig. 4) used for this purpose in the thesis is outlined as follows:

First, the quality of the raw sequencing reads was assessed using FASTQC (Andrews, 2010), followed by adapter trimming with Trimmomatic (Bolger, Lohse and Usadel, 2014). Contaminants were then identified and removed using KAT (Mapleson *et al.*, 2017) and BlobTools (Laetsch and Blaxter, 2017). The genomes were assembled using SPADES (Prjibelski *et al.*, 2020) in the

metagenomic mode and annotated with the funannotate pipeline (Palmer and Stajich, 2023). Specific genes, such as CAZymes, peptidases and lipases, were annotated using dbCAN2 (Yin *et al.*, 2012) with the CAZy database for CAZymes, funannotate with the MEROPS database for peptidases and BLAST against the LED database for lipases (http://www.led.uni-stuttgart.de). The orthologous genes were inferred using OrthoFinder (Emms and Kelly, 2019). The resulting single copy orthologues were aligned using MAFFT (Katoh and Standley, 2013) and these alignments were trimmed using trimAl (Gutierrez, Martínez and Gabaldón, 2009). Concatenation based species tree and individual gene trees were drawn using IQ-TREE (Nguyen *et al.*, 2015). A coalescent-based species tree was built using ASTRAL-III (Mirarab and Warnow, 2015). The comparative genomic analysis (gene family evolution analysis) was performed using CAFE4 (Han *et al.*, 2013) and genomics features of the different species were compared using the gene copy numbers of CAZymes, peptidases and lipases.

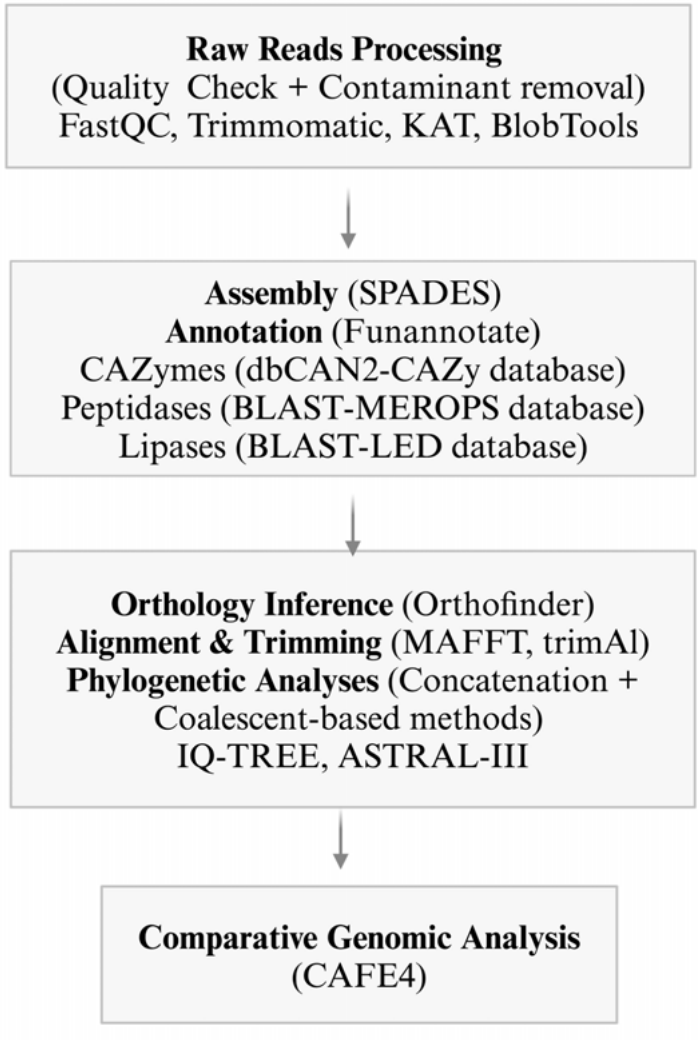

Figure 4. Generalized phylogenomics workflow fully or partly used in **Papers II, III** and **IV**.

Model-based hypothesis testing

In the comparative phylogenetic framework, different questions about the evolutionary history of organisms, such as how often these organisms transition to and from a specific lifestyle, whether these transitions occur at different rates across lineages or during different geological time periods (Blomberg, Garland Jr and Ives, 2003), can be formulated as models and their parameters (O'meara *et al.*, 2006; Burns, Murphy and Zheng, 2019).

The parameters of the models can be adjusted to represent different hypotheses, for example, by adding or restricting them, and these models, representing different hypotheses, can then be compared to determine which one best explains the observed data (Ryberg, Kalsoom and Sánchez-García, 2022). Since models are simplified representations of evolutionary processes, they can be better or worse reflections of biological reality. This is why, testing multiple models and comparing the results is very important as it influences our understanding of the underlying biological process (**Paper I**).

Besides model-testing, the model's ability to explain the observed patterns can also be evaluated by generating random datasets under the same model assumptions through simulations. If the observed data closely matches to what the model predicts, the model can be considered a good fit, which suggests the hypothesized evolutionary process is likely (Boettiger, Coop and Ralph, 2012). These model-based methods have been used to test different hypotheses in **Papers I** and **II.**

AIMS

The overarching goal of this thesis is to improve our understanding of the origin and diversification of ECM symbiosis. And the specific objectives are:

- To test the different hypotheses regarding the origin and evolution of ECM fungi using different models, including variable rate models through time and lineages
- To investigate whether the previously observed ECM-related genomic changes e.g contractions in CAZymes, occurred abruptly at the transition to the ECM lifestyle or gradually throughout the evolution of ECM lineages
- To investigate whether the number of lineages or the number of species within the lineage influences the functional diversity of ECM fungi
- To explore the evolution of genes involved in the synthesis of psilocybin in Inocybaceae species

Summary of the papers

Paper I

ECM fungi are one of the highly diverse groups of organisms in fungi, with over 20 000 species. Previous studies have shown that there were multiple independent origins of ECM symbiosis from diverse types of saprotrophs such as white-rot, brown-rot and soil/litter decaying fungi. Conflicting views exist regarding how this diversity of ECM fungi arose i.e. (i) there were few transitions to the ECM lifestyle, followed by many reversals to their ancestral lifestyle of saprotrophy; (ii) there were multiple independent origins of ECM lineages with no reversals to saprotrophy; (iii) different time periods, such as the timing of the origin of their host plants, were important for the diversification of these lineages. Few studies have tested these hypotheses using comprehensive datasets of ECM lineages and appropriate models.

In this study, a megaphylogeny based on an inclusive dataset of 2 174 taxa from different ECM lineages using five marker genes (nucLSU, nuc5.8S, nucSSU, RBP1 and RPB2) was built, to test different hypotheses related to the evolution of ECM using model-based methods. The main hypotheses tested include (i) whether there were reversals to the saprotrophic lifestyle from ECM or not, and (ii) whether different geological time periods were important for the evolution of ECM fungi. Models with a zero transition rate from ECM to non-ECM states and models with equal transition rates between ECM and non-ECM states were compared in order to test the hypothesis that there have been no reversals to the saprotrophic lifestyle. Models with different transition rates in different geological time periods, models with different rates in several clades of ECM, and models with constant rates throughout the tree were also compared using AIC (Akaike information criterion), and the fit of all these models to the data was assessed using simulations.

The findings in this study show that the non-reversible model with rate shifts in clades is the best fit model to our data. The models that allowed rate shifts in clades and time overall fit the data better compared to constant rate models. The results further show that rate shifts in clades are more important than the rate shifts in time, as all models that implemented rate shifts in clades have the lowest AIC score. Our findings suggest that the reversals to saprotrophy are probably rare, if they occur at all. The results also suggest that our conclusions on the relative transition rate between ECM and saprotrophy depend on whether rate shifts are allowed in the models, as we find that constant

rate models support reversals to saprotrophy whereas variable rate models do not. We conclude that simple models, assuming a constant rate of evolution through the tree, can sometimes give results that are likely to be incorrect. Caution is therefore exercised against the simplistic use of such models for understanding the evolution of complex traits like ECM.

Paper II

Previous studies have highlighted a number of characteristic genomic changes in ECM lineages, which include contractions of genes encoding Carbohydrate Active enzymes (CAZymes) and expansions in Transposable Elements (TEs) and mycorrhiza-specific small secreted proteins compared to their saprotrophic relatives. A reduced number of genes encoding specific peptidases (serine peptidases) and lipases (versatile lipases) has also been reported. It is still unclear whether these changes occurred abruptly in proximity to the transition to the ECM lifestyle or gradually throughout the evolution of ECM lineages.

A phylogenomic tree based on whole-genome data from 20 ECM fungal species within a single ECM lineage, Inocybaceae, along with extended saprotrophic outgroups including the sister lineage Crepidotaceae was built, and comparative genomic analyses were carried out to investigate whether previously observed ECM-related genomic changes occurred abruptly along the branch associated with the transition to the ECM lifestyle or not.

We recovered near complete genomes of the species sequenced in this study, with up to 94% BUSCO scores using fungal material from dried sporocarps. We presented the first phylogenomic tree of Inocybaceae family and confirmed the monophyly of the Inocybaceae lineage, as well as the genera within this lineage. The Inocybaceae genomes showed molecular signatures of the ECM lifestyle and had lower gene sets for CAZymes, peptidases, lipases, secondary metabolite gene clusters and small secreted proteins, and higher TE content relative to their saprotrophic relatives. Our analyses showed that there was a high rate of gene family evolution along the transition branch to the ECM lifestyle. The contractions in CAZymes also occurred at a very high rate along this branch. Furthermore, this branch had significant contractions of saprotroph-specific peptidases, as well as expansions in transposons, transport-related genes, and communication genes like fungal kinases among others, which suggests that the symbiosis toolkit needed for the ECM lifestyle evolved largely in close proximity to the transition to this lifestyle.

Paper III

ECM fungal communities are usually highly diverse, consisting of hundreds of species. ECM fungi have evolved multiple times independently into distinct lineages. These fungi play an important role in nutrient cycling by breaking down complex compounds in their environment through the action of their secreted enzymes such as CAZymes (Carbohydrate Active enZymes), peptidases and lipases. Previous studies have shown that higher ECM fungal species richness is associated with increased enzymatic activity in soil. With the growing availability of sequenced genomes from different ECM fungal lineages, the genomic and functional diversity of these fungi is being revealed. However, many of these studies have focused either on a single ECM lineage when many species were included or on the similarities and differences between lineages when species from different lineages were included, resulting in a lack of a comparative perspective that addresses diversity within and between lineages.

A phylogenetically diverse dataset of 75 species from eight different lineages of ECM fungi was generated, and phylogenomic and statistical analyses were performed, to investigate whether the number of lineages or the number of species within a lineage contributes more to the functional diversity of ECM lineages.

The phylogenomic tree, based on 470 single copy BUSCO genes, showed distinct clades for each lineage, with genera positioned as expected within their respective lineages. The statistical analyses showed that the addition of certain lineages, such as /tuber-helvella, increases the diversity in gene families of CAZymes and peptidases. Although individual lineages may contribute specific functions, we found no strong correlation between the number of lineages and overall gene family diversity beyond the inclusion of a few lineages. We also found that certain individual species can have a large impact on the functional diversity, for example, *Amanita fuliginea* in the case of peptidases. For lipases, sampling from certain individual lineages can result in higher average diversity than sampling species across all lineages. Furthermore, while functional diversity levels off with the addition of more lineages, it continues to increase with the addition of more species. These results suggest that species richness is important for shaping the ECM functional diversity, whereas for lineages it is the inclusion of particular lineages, rather than their overall number, that has the greatest impact.

Paper IV

Psilocybin, a psychedelic compound, has recently received significant attention because of its pharmaceutical applications. This hallucinogen is produced by mushroom-forming fungi of various genera in Agaricales, including

Psilocybe, Gymnopilus, Panaeolus and *Inocybe* (Inocybaceae). The psilocybin gene cluster has been found to have originated in *Psilocybe* species and was transferred via horizontal gene transfer to species in other genera. A distinct gene cluster for psilocybin was recently identified in *I. corydalina*, a psilocybin producing species of Inocybaceae. Inocybaceae species, suggested to produce psilocybin, belong to three different clades. Notably, all known psilocybin producing species in Inocybaceae have been tested negative for muscarine (another common toxin produced by species in this family), suggesting that these two toxins are mutually exclusive in species of this family. However, it is unclear whether this pattern is consistent as only a few species in Inocybaceae have been screened for both toxins. Additionally, the origin and evolution of the genes involved in psilocybin synthesis in Inocybaceae are still unknown.

To test the idea of mutual exclusivity of psilocybin and muscarine in Inocybaceae species, 21 mushroom samples were chemically screened for the presence of these toxins. To study the origin and evolution of psilocybin genes in Inocybaceae, a dataset consisting of 24 whole genomes from Inocybaceae species was generated. Two species, suggested to produce psilocybin, from two different clades i.e *Inocybe aeruginascens* and *Inosperma calamistratum* were sequenced in this study and another species from the third clade i.e., *Inocybe corydalina* together with 21 other Inocybaceae species were downloaded from the NCBI's GenBank. The psilocybin gene cluster in the genomes was annotated using a custom pipeline based on reciprocal best blast hit method by blasting all genes from the whole dataset against a database containing genes from the psilocybin cluster in *I. corydalina*.

Our chemical analysis detected psilocybin and muscarine in *I. asterospora*, suggesting that these two toxins may not be mutually exclusive as previously thought. The phylogenomic tree showed the positions of all Inocybaceae species in the tree as expected. The phylogenetic analyses of each core gene involved in psilocybin production showed distinct clades of Inocybaceae species, suggesting that there may not have been horizontal gene transfer of the psilocybin gene cluster from other genera into Inocybaceae. We found complete or partial psilocybin gene cluster in several other species of Inocybaceae apart from *I. corydalina*, which suggests that this cluster is more widespread in Inocybaceae species. Our study also suggests that the psilocybin gene cluster in Inocybaceae has evolved through convergent evolution with two different origins, as we did not find the same gene cluster in *I. aeruginascens* as in *I. corydalina*.

Concluding remarks

To conclude, this thesis highlights the complexity of the evolution of traits such as ECM symbiosis and psilocybin production and emphasizes that functional similarity does not always imply a shared origin.

Testing the hypotheses regarding ECM evolution (whether there were multiple independent origins of ECM lineages or there were reversals to the ancestral lifestyle of saprotrophy), with both variable and constant rate models and a comprehensive dataset, has drawn our attention to how model assumptions can influence our understanding of the evolution of ECM fungi and how difficult it is to get clear answers regarding the evolution of complex traits like ECM despite using an extensive dataset.

Comparative genomics of ECM Inocybaceae and saprotrophic Crepidotaceae helped shed light on important molecular changes occurring in proximity to the transition to ECM in one of the diverse lineages of ECM. This study highlighted the genomic features in Inocybaceae species that are similar to previously sequenced genomes of ECM species but also features unique to species in this lineage.

Analysing the genomic diversity of ECM lineages through their secretomes provided the first insights into the relative contributions of the number of lineages and the number of species within the lineage to the overall functional diversity of ECM fungi.

Phylogenetic analyses of the core genes involved in psilocybin production revealed convergent evolution of this psychedelic compound in Inocybaceae as well as the potential two different origins of the gene cluster within this family. This study also showcased the unique instance of the coexistence of psilocybin and muscarine in Inocybaceae.

Future perspectives

When I first started my PhD in 2017, only a handful of genomes from ECM species were published. The main ECM comparative genomic study published at the time was by Kohler *et al.* (2015), which included 13 ECM species from different lineages. The ECM genomics era was just beginning, and no comparative genomics study had been published that focused on a single lineage. It was incredibly exciting to follow the genomic advancements in ECM fungi throughout the years of my PhD studies. Research groups from around the world published several studies focusing on different ECM fungal lineages during these years, starting with comparative genomic study of Amanitaceae lineage in 2018 (Hess *et al.*, 2018), Tuberaceae also in 2018 (Murat *et al.*, 2018), Endogonaceae in 2019 (Chang *et al.*, 2019), Suillaceae in 2021 (Lofgren *et al.*, 2021), *Lactarius* (Russulaceae) also in 2021 (Lebreton *et al.*, 2021), Russulaceae in 2022 (Looney *et al.*, 2022b) and Boletaceae also in

2022 (Wu *et al.*, 2022), *Pisolithus* (Sclerodermataceae) in 2023 (Plett *et al.*, 2023) and most recently my study on Inocybaceae in 2024 (**Paper II**). With each of these published papers, our understanding of the molecular evolution of ECM symbiosis has grown. These studies have mainly aimed to unravel the common genomic features of ECM species. However, what I found most fascinating was that these different ECM lineages have more genomic differences than similarities, each evolving its own unique, independent strategies for interacting with host plants.

The number of ECM genomes in the public databases is growing enormously with each passing year, largely due to the JGI's fungal genome sequencing projects. Contributing to these efforts, we sequenced 54 new fungal genomes in this thesis, 52 of which cover five key ECM lineages. This growing genomic resource opens up opportunities to address many important questions regarding the ecology and evolution of ECM fungi. I am confident that more genomes from previously unstudied ECM lineages will become available in the coming years, and I especially look forward to the sequencing and availability of the genomic data from some poorly explored lineages such as those in Sebacinales and Thelephorales and the new insights they will bring to our understanding of molecular ECM symbiosis.

In the future, it would be interesting to investigate the evolutionary dynamics of TEs in Inocybaceae species compared to their close saprotrophic relatives as TEs play a key role in genome expansions and other genomic innovations in the ECM lineages and Inocybaceae genomes are smaller than most of the other ECM species. Furthermore, new annotation methods are being developed to annotate transporter proteins such as nitrogen and phosphate transporters in mycorrhizal fungi and the evolution of these transporters in ECM lineages would be an exciting avenue to explore in the future given the major role of these proteins in nutrient acquisition in the ECM fungi. Since Inocybaceae species are rich in a number of toxic metabolites, including muscarine and aeruginascin (exclusive to *I. aeruginascens*), the newly available multiple Inocybaceae genomes provide an opportunity to characterize the biosynthetic gene clusters responsible for these toxins and to explore their evolutionary history in this lineage. It would also be valuable to examine the genomic diversity of ECM fungi at the community level by sampling and sequencing species belonging to different lineages within the same community of ECM and comparing this to the diversity between different communities, rather than analysing genomes of ECM species from different lineages that are not part of the same community. Last but not least, it would be pertinent to assess whether the expanded gene families in Inocybaceae are transcriptionally active or not.

Before I close, I would also mention a few general important lessons that I learned, while working on fungal genomics and evolution for several years during my PhD. Firstly, proper filtration of contaminants is crucial when using fungal sporocarps for DNA extractions and also rigorously quality checks are needed when working with publicly available data. It was surprising for me to

see up to 70% BUSCO gene duplicates in publicly available genomes, which had been used to answer important questions in different publications and yet this was never mentioned in those studies. Secondly, it was interesting for me to learn that using the same technique or DNA sequencing technology may not be suitable for all studies. For example, using dried sporocarps with Illumina sequencing worked well for gene family evolution analysis in **Paper II** but was less effective for secondary metabolite gene cluster analysis in **Paper IV**. Thirdly, careful model testing is necessary, even in comparative genomic analyses using CAFE, where the users often overlook model selection and default models are assumed to be a good fit.

Svensk sammanfattning

Föreställ dig att du promenerar genom en frodig skog och beundrar de imponerande träden och det sprudlande ekosystemet omkring dig. Vad du kanske inte inser är att under jordytan pågår det ett fascinerande samspel mellan träden och en grupp svampar som bildar så kallad ektomykorrhiza (ECM). Detta är ett ömsesidigt, fördelaktigt partnerskap mellan svampar och träd, där svamparna får energirika ämnen från träden i utbyte mot livsnödvändiga näringsämnen som kväve, fosfor och vatten. Denna relation är avgörande för skogsekosystemet, då den underlättar kretsloppet av näringsämnen och främjar växternas tillväxt. Men hur uppstod denna anmärkningsvärda relation? Tidigare studier visar att ECM-svampar, som en gång var nedbrytare av dött trä, förna och jord (saprotrofa svampar), i flera olika utvecklingslinjer övergått till att bli mutualister med växter.

Artikel I

Inom forskningen har man diskuterat huruvida ECM-svampar någonsin återgår till en saprotrof (nedbrytande) livsstil, eller om den evolutionära övergången till ECM är permanent, när den en gång skett. För att undersöka detta skapade vi ett omfattande evolutionärt träd med 2 174 ECM-arter baserat på fem nyckelgener. Vi testade olika modeller för att förstå ECM-evolutionen, bland annat: 1) Om ECM-svampar någonsin återgått till saprotrofi. 2) Om olika geologiska perioder påverkat evolutionen av ECM. Våra resultat tyder på att ECM-svampar sällan, om ens någonsin, återgår till att vara nedbrytare. Vi testade flera evolutionära modeller, även sådana där utvecklingen sker med olika hastigheter i olika svampgrupper och över tid, istället för i en konstant takt. Det visade sig att förändringar i evolutionstakten mellan olika svampgrupper (klader) var viktigare för ECM: s utveckling än förändringar över geologisk tid. En viktig slutsats från studien är att enkla modeller med fasta utvecklingshastigheter kan ge missvisande resultat – dessa modeller föreslog felaktigt att ECM-svampar ofta återgick till saprotrofi. Studien understryker vikten av att använda flexibla modeller när man undersöker evolutionen av komplexa egenskaper såsom ECM-symbios.

Artikel II

Genom att jämföra genom hos ECM-svampar och saprotrofer har man upptäckt att ECM-svampar genomgått betydande genetiska förändringar, inklusive förlust

av gener som kodar för enzymer som t.ex. CAZymer – enzymer de tidigare, när de var saprotrofer, använde för att bryta ner växtcellväggar. De har också utvecklat nya gener, som kodar för så kallade ”small secreted proteins”, som är nödvändiga för att interagera med värdväxter. Detta tyder på att ECM-svampar har gjort sig av med ursprungliga gener som inte längre behövs, och istället utvecklat nya gener som är viktiga för att etablera symbios med växter. Men när skedde dessa genetiska förändringar? Var det ett plötsligt skifte vid övergången till ECM-livsstilen, eller en gradvis process? För att besvara detta sekvenserade vi genom från flera arter inom en grupp av ECM-svampar med stor mångfald och jämförde dem med sina närmaste saprotrofa släktingar. Våra resultat visade att de flesta av de genetiska förändringarna kopplade till levnadssättet som ECM-arter skedde precis vid övergången till denna symbiotiska sammankoppling, vilket tyder på att förlust eller strikt reglering av vissa ursprungliga gener är avgörande för att kunna anta detta viktiga levnadssätt.

Artikel III

ECM-svampsamhällen har vanligtvis en otrolig mångfald, ofta bestående av hundratals arter. Trots att forskare har studerat ECM-svampar i många år förstår vi fortfarande inte helt hur deras mångfald påverkar de ekosystem de stöder. Tack vare framsteg inom genom-sekvensering har vi idag fler verktyg än någonsin för att utforska den genetiska och funktionella mångfalden hos dessa svampar. De flesta genom-baserade studier har dock hittills fokuserat på en enskild grupp av närbesläktade arter, vilket innebär att jämförelser mellan olika linjer för att förstå helheten fortfarande saknas. I denna studie skapade vi ett stort och varierat dataset med 75 ECM-arter från åtta olika evolutionära linjer. Med hjälp av avancerade genom-baserade och statistiska metoder hade vi för avsikt att besvara en viktig fråga: är det viktigare att ha många evolutionära linjer eller många arter inom en linje för att uppnå hög genetisk mångfald? Vi fann att vissa linjer, som /tuber–helvella, hade en märkbar effekt på mångfalden av specifika enzymer, såsom CAZymer och peptidaser, som svampar använder för att bryta ner komplexa organiska material i jorden. Men att lägga till fler linjer ledde inte alltid till ökad genetisk mångfald. Faktum är att mycket av mångfalden täcktes in efter att bara några få evolutionära linjer inkluderades. Vissa enskilda arter stack dock ut – exempelvis bidrog Amanita fuliginea särskilt starkt till mångfalden av peptidaser. Och när det gällde lipaser, enzymer som bryter ner fetter, kunde inkluderande av endast en linje ibland bidra med mer genetisk mångfald än inkluderandet av alla linjer tillsammans. En av våra viktigaste slutsatser var att även om inkluderandet av fler evolutionära linjer till slut leder till en platå i funktionell mångfald, fortsätter inkluderandet av fler arter att bidra med nya gener och funktioner.

Artikel IV

Psilocybin, det psykoaktiva ämnet i "magiska svampar", har fascinerat både forskare och allmänhet. Ny forskning visar att Inocybe corydalina, en psykedelisk svamp inom familjen Inocybaceae, producerar psilocybin med hjälp av ett helt annat genkluster än andra magiska svampar. Men varifrån kommer de gener som ansvarar för psilocybinproduktionen inom Inocybaceae? Vi undersökte detta genom att analysera genom från olika Inocybaceae-arter, varav några producerar psilocybin. Vi genomförde även kemiska tester av 21 prover från familjen för att undersöka förekomsten av både psilocybin och muskarin (ett annat vanligt toxin från dessa svampar) – eftersom man tidigare trodde att en art inte kunde producera båda. Vår studie visar att psilocybin-generna sannolikt har uppstått inom Inocybaceae och inte kommit från andra arter (så kallad horisontell genöverföring). Även om det tidigare var känt att I. corydalina bar på psilocybin-genklustret, hittade vi hela eller delar av klustret i flera andra arter inom familjen, vilket tyder på att dessa gener är mer utbredda än man tidigare trott. Vi upptäckte att I. aeruginascens inte har samma genkluster som I. corydalina, vilket antyder att psilocybinproduktionen har utvecklats åtminstone två gånger inom familjen. Dessutom visade våra kemiska analyser att både psilocybin och muskarin kan förekomma i samma art – närmare bestämt Inocybe asterospora. Detta utmanar den tidigare uppfattningen att Inocybaceae-arter antingen producerar det ena giftet eller det andra, men inte båda.

خلاصہ برائے پاپولر سائنس

آپ آنکھیں بند کرکے تھوڑی دیر کے لیے یہ تصور کریں کہ آپ ایک سر سبز اور شاداب جنگل سے گزر رہے ہیں اور اس دوران آسمان کو چھوتے ہوئے درختوں اور اس کے ارد گرد کے ایکو سسٹم کا مشاہدہ کر رہے ہیں۔ لیکن آپ اس حقیقت سے بے خبر ہیں کہ زمین کی تہہ میں چھپے ہوئے ایک پھپندی کے گروہ جیسے ایٹموکوریہیزل کہا جاتا ہے اور اردگرد کے درختوں کے درمیان ایک بہت گہرا تعلق ہوتا ہے۔یہ ایک باہمی فائدے کی شرکت داری ان دونوں عناصر کے درمیان پائی جاتی ہے جس میں پھپندی درختوں سے توانائی سے بھرپور مرکبات حاصل کرتی ہے اور بدلے میں درختوں کو نائٹروجن ،فاسورس اور پانی دیتی ہے۔

ان کا یہ باہمی تعلق جنگل کے ماحولیاتی نظام کے لیے نہایت اہم ہے، اس لئے کہ یہ غذائی اجزاء کی پیداوار میں مدد دیتا ہے اور پودوں کی نشوونما کو بہتر بناتا ہے۔ لیکن سوال یہ پیدا ہوتا ہے کہ یہ حیرت انگیز تعلق کیسے وجود میں آیا؟

Iمقالہ

ماضی میں کیے گئے تحقیق سے معلوم پڑتا ہے

کہ ای سی ایم فنگس، جو کبھی مردہ لکڑی، پتوں اور مٹی کو گلانے والے سپروٹروپیکل فنگس تھے، مختلف فنگل اقسام میں آزادانہ طور پر پودوں کے ساتھ باہمی تعلق میں تبدیل ہو گئے۔

سائنسدانوں میں یہ بحث عام رہی ہے کہ آیا ای سی ایم فنگس کبھی واپس گلنے والے سپروٹروپیکل طرز زندگی کی طرف لوٹے ہیں یا ایک بار ای سی ایم میں تبدیلی کے بعد یہ مستقل تبدیلی بن جاتی ہے۔ اس کی جانچ کے لیے، ہم نے 2,174 ای سی ایم فنگس انواع کا ایک بڑا ارتقائی شجر پانچ اہم جینز کے استعمال سے بنایا۔ ہم نے مختلف ماڈلز کی جانچ کی تاکہ یہ سمجھا جا سکے کہ:

کیا ای سی ایم فنگس کبھی واپس گلنے والے فنگس بنے؟

مختلف ارضیاتی ادوار نے ای سی ایم ارتقا کو کس طرح متاثر کیا؟

ہمارے نتائج بتاتے ہیں کہ ای سی ایم فنگس شاذ و نادر ہی دوبارہ گلنے والے فنگس میں تبدیل ہوتے ہیں۔ ہم نے مختلف ارتقائی ماڈلز آزمائے، بشمول وہ ماڈلز جو ارتقا کی شرح کو مختلف فنگل گروپوں اور وقت کے ساتھ مختلف ہونے کی اجازت دیتے ہیں، بجائے اس کے کہ یہ مستقل رفتار سے ہو۔ درحقیقت، ہم نے پایا کہ فنگل گروپوں کلیڈز کے درمیان ارتقائی تبدیلیوں کی شرحیں ارضیاتی ادوار کے مقابلے میں زیادہ اہم تھیں۔

ہمارے مطالعے کا ایک اہم نتیجہ یہ ہے کہ سادہ ماڈلز، جن میں ارتقائی شرحیں مستقل فرض کی جاتی ہیں، غلط نتائج دے سکتے ہیں—یہ ماڈلز غلط طور پر یہ تجویز کرتے ہیں کہ ای سی ایم فنگس اکثر واپس سپٹروپی کی طرف چلے جاتے ہیں۔ یہ تحقیق پیچیدہ خصوصیات جیسے ای سی ایم سیمبیاسس کے مطالعے کے لیے لچکدار ماڈلز کے استعمال کی اہمیت کو اجاگر کرتی ہے۔

IIمقالہ

ای سی ایم فنگس کے جینومز کا سپروٹروپیک فنگس کے ساتھ موازنہ کرتے ہوئے، محققین نے دریافت کیا کہ ای سی ایم فنگس میں نمایاں جینیاتی تبدیلیاں واقع ہوئی ہیں، بشمول کیزیم جیسے انزائمز کو کوڈ کرنے والے جینز میں کمی، جو انہیں ماضی میں پودوں کی خلیاتی دیواروں کو ہضم کرنے کے لیے درکار تھے۔

اس کے علاوہ، انہوں نے نئے جینز حاصل کیے ہیں، جنہیں "چھوٹے خفیہ شدہ پروٹینز کہا جاتا ہے، جو ان کے میزبان پودوں کے ساتھ تعلق قائم کرنے کے لیے ضروری ہوتے ہیں۔ اس سے ظاہر ہوتا ہے کہ ای سی ایم فنگس نے وہ آبائی جینز چھوڑ دیے جو ان کے نئے طرز زندگی میں غیر ضروری ہو گئے، اور اس کے بجائے وہ جینز حاصل کیے جو میزبان پودوں کے ساتھ تعلق قائم کرنے کے لیے اہم تھے۔

لیکن یہ جینیاتی تبدیلیاں کب واقع ہوئیں؟ کیا یہ تبدیلی اچانک ہوئی جب فنگس ای سی ایم طرز زندگی میں داخل ہوئے، یا یہ بتدریج وقت کے ساتھ ہوا؟ اس سوال کا جواب جاننے کے لیے، ہم

نے ای سی ایم فنگس کی مختلف اقسام کے جینومز ترتیب دیے اور ان کا ان کے قریبی سپٹروپیکل رشتہ داروں کے ساتھ موازنہ کیا۔
ہمارے نتائج نے ظاہر کیا کہ ای سی ایم طرز زندگی سے وابستہ زیادہ تر جینیاتی تبدیلیاں بالکل اسی وقت واقع ہوئیں جب یہ سیمبیاٹیک تعلق قائم ہوا۔ اس سے ظاہر ہوتا ہے کہ آبائی جینز کا ضیاع یا ان کا سخت کنٹرول اس اہم ماحولیاتی کردار کو اپنانے کے لیے ضروری ہے۔ یہ تحقیق ای سی ایم فنگس کی ابتدا سے متعلق ارتقائی واقعات پر روشنی ڈالتی ہے اور اس پیچیدہ باہمی تعلق کو سمجھنے میں مدد دیتی ہے۔

IIIمقالہ

ای سی ایم فنگس کی کمیونٹیز عام طور پر بے حد متنوع ہوتی ہیں، جن میں اکثر سیکڑوں انواع شامل ہوتی ہیں۔ اگرچہ محققین برسوں سے ای سی ایم فنگس کا مطالعہ کر رہے ہیں، لیکن ہم اب بھی پوری طرح نہیں سمجھ پائے کہ ان کی حیاتیاتی تنوع ماحولیاتی نظام پر کیسے اثر انداز ہوتی ہے۔
جینوم ترتیب دینے میں ہونے والی ترقی کی بدولت، ہمارے پاس اب ان فنگس کی جینیاتی اور عملیاتی تنوع کو دریافت کرنے کے پہلے سے زیادہ مواقع موجود ہیں۔ تاہم، زیادہ تر جینیاتی مطالعات نے صرف ایک گروہ کی قریبی انواع پر توجہ دی ہے، اور مختلف اقسام کے درمیان تقابلی تجزیہ ابھی تک مکمل طور پر نہیں کیا گیا ہے۔
اس تحقیق میں، ہم نے 74 ای سی ایم فنگس انواع کا ایک بڑا اور متنوع ڈیٹا سیٹ تیار کیا، جو آٹھ مختلف ارتقائی سلسلوں سے تعلق رکھتی تھیں۔ جدید جینومی اور شماریاتی طریقوں کا استعمال کرتے ہوئے، ہم نے یہ اہم سوال حل کرنے کی کوشش کی: کیا زیادہ اقسام کا ہونا زیادہ اہم ہے یا زیادہ ارتقائی سلسلے کا؟
ہم نے پایا کہ کچھ سلسلے، جیسے ٹیوبر-ہیلوویلا", مخصوص انزائمز کے تنوع پر واضح اثر ڈالتے ہیں، جیسے کاربوہائیڈریٹ-فعال خامرے اور پیپٹائیڈیز، جو فنگس مٹی میں پیچیدہ نامیاتی مواد کو توڑنے کے لیے استعمال کرتے ہیں۔ تاہم، زیادہ سلسلوں کا اضافہ ہمیشہ جینیاتی تنوع میں اضافہ نہیں کرتا۔ درحقیقت، ہم نے پایا کہ زیادہ تر تنوع چند ارتقائی سلسلوں کے شامل ہونے کے بعد حاصل کیا جا سکتا ہے۔
دلچسپ بات یہ ہے کہ بعض انواع نے بھی نمایاں کردار ادا کیا، جیسےایمانیٹافولیجینیا ، جس نے پیپٹائیڈیز تنوع میں خاصا اضافہ کیا۔ اور لپیز کے معاملے میں، جو چکنائی کو توڑنے میں مدد دیتے ہیں، ایک ہی سلسلے کی چند انواع بعض اوقات تمام سلسلوں کو ملا کر حاصل کیے گئے تنوع سے زیادہ جینیاتی تنوع فراہم کر سکتی ہیں۔
ہمارا ایک اہم نتیجہ یہ تھا کہ اگرچہ زیادہ سلسلوں کو شامل کرنے سے آخرکار تنوع ایک حد پر پہنچ جاتا ہے، لیکن زیادہ انواع کو شامل کرنے سے مسلسل نئے جینز اور افعال دریافت ہوتے ہیں۔

IVمقالہ

سیلو سایبین، وہ سائیکوڈیلیک مرکب جو "جادوئی مشرومز" میں پایا جاتا ہے، طویل عرصے سے سائنسدانوں اور عوام کی دلچسپی کا مرکز رہا ہے۔ حالیہ تحقیق سے معلوم ہوا ہے کہ اینو سایبی کرویڈلنا, جو کہ اینوسایبائ خاندان کی ایک سائیکوڈیلیک مشروم ہے، سیلو سائبین بنانے کے لیے جینیاتی طور پر مختلف راستہ اپناتی ہے۔
لیکن اینو سائباسی میں سائلوسائبن پیدا کرنے والے جین کہاں سے آئے؟ اس سوال کا جواب جاننے کے لیے، ہم نے اینو سائباسی خاندان کے مختلف فنگس کے جینومز کا تجزیہ کیا، جن میں سے کچھ سائلوسائبن پیدا کرتے ہیں۔ ہم نے 21 اینو سائباسی نمونوں کا کیمیائی تجزیہ بھی کیا تاکہ معلوم کیا جا سکے کہ کیا ایک ہی نوع دونوں زہریلے مرکبات، سائلوسائبن اور مسکارین, پیدا کر سکتی ہے۔

حیرت انگیز طور پر، ہمارے کیمیائی تجزیے میں معلوم ہوا کہ اینو سائبے ایسٹیروسپورا میں دونوں مرکبات موجود ہیں، جو اس پہلے کے مفروضے کو چیلنج کرتا ہے کہ اینو سائباسی میں یا تو ایک زہر پیدا ہوتا ہے یا دوسرا، مگر دونوں نہیں۔
ہماری تحقیق سے یہ بھی معلوم ہوا کے سائلوسائبن بنیادی جینز اینو سائباسی کے اندر ہی ارتقا پذیر ہوئے، نہ کہ کسی بیرونی ذریعے سے آئے۔ مزید برآں، ہم نے پایا کہ سائلوسائبن کی پیداوار کم از کم دو بار اس فنگل خاندان میں آزادانہ طور پر ارتقا پذیر ہوئی ہے۔ یہ تحقیق اینو سائباسی میں سائلوسائبنجینز کے ارتقا پر روشنی ڈالتی ہے۔

Acknowledgements

Supervisors: First of all, I would like to thank my main supervisor, **Martin**, for selecting me for this PhD position out of 52 applicants. Thank you for your deep commitment to research ethics and for being so meticulous and detail-oriented in your work. I have learned so much from you. I am truly grateful to you for your immense support and guidance throughout this journey. Thank you for your trust in me, for your patience over the years and for answering my countless (and sometimes silly) questions. I am especially thankful for the academic freedom you gave me in all my PhD projects and for allowing me to add new components to the projects that I was interested in. Thank you for being so generous in supporting my participation in conferences, workshops and courses. And, thank you for the many opportunities you provided to teach, take part in administrative tasks and get involved in side-projects during my studies period. I really appreciate the long forest-walk meetings that you arranged with baby Elias during your parental leave. You are the kind of supervisor everyone wishes they had :-) **Hanna**, my co-supervisor, thank you for your constructive comments on the manuscripts, for the energy you brought to our group meetings and for always adding a new angle to our research discussions. Everything just made more sense to me after you said it :-) Your encouraging words at the end of each meeting were always a breath of fresh air. **Marisol**, my second co-supervisor, thank you for being super nice, always responding quickly to my emails and guiding me through different analyses. I like your thoughtful feedback and have been a big fan of the comments you gave on anything related to my PhD :-) **Anna Rosling**, though you were not officially my supervisor in my PhD studies but, I think, it is important to acknowledge you here as you were the reason, I got interested in fungi in the first place. Thank you for being so considerate, inspirational, fun-loving and passionate about fungi! Your energy is contagious. Thank you for introducing me to the mesmerizing world of fungi and for the general great conversations about science, life and the fun times during ECFG16, ICOM12 and IMC12. Thank you also for the mentorship for my career after PhD!

Past and present members of **Ryberg** group: Thank you, **Brendon**, for being always ready to help- whether it was during mushroom hunting, lab work, teaching or providing information on almost anything. Thank you, **Bobby**, for the nice time during the field work in Öland. Thank you **Sten** for always being so cheerful, for the discussions about Pakistani politics, for the calls for fika

and for spreading smiles in the entire program. Also, for the Swedish translation of the summary. **Xi-Hui**, for the nice chats over fikas and lunches. **Mohammed**, thank you for your enthusiasm about fungal research and for the helpful discussions on different topics related to fungi. Thank you for encouraging me to apply for this PhD position. I would not have applied for it if it weren't for you.

Past and present members of **SystBio**: **Petra**, it is always a pleasure to talk to you. The blanket handmade by you is so impressive! :-) **Tobias**, thank you for being so kind and cordial. By the way, do you wanna have a cake? :-) **Yash**, thank you for introducing me to ChatGPT and for radiating positive energy :-), **Nina**, I like your carefree nature. It's a good reminder that sometimes it's okay not to take things too seriously :-) **Monica**, thank you for the nice chitchats here and there and for the idea of starting writing earlier than later. **Adrian Baggström**, Good luck with the OEM course, you will be an expert on fungi by the end :-) Thank you, **Jenni** for your amazing sense of humor and **Sandie**, for teaching me phylogenetics during the phylogenetics course :-) and also for always being so lively. **Ioana**, it is always good to talk to you. **Markus**, thank you for your sound advices related to fungal research and also the fieldwork. **Aaron**, thank you for the greetings with a smile in the SystBio corridor. **Nahid**, thank you for being so caring and also for your help in the lab and also for the general discussions about life. **Jakob**, thank you for your help with the Swedish translation of the summary. Thank you, **Xuan**, **Fabien**, **Debora**, **Anders**, **Raquel**, **Anna Miradola** and **Karin Steffen**, for the nice discussions over lunches. Thank you to all the other past and present members of SystBio for making this program an amazing place to work at!

Admins: Big thank you to all the admin staff i.e **Karin**, **Yvonne**, **Matilda**, **Iva**, **Tonja** and **Nina** for your prompt responses to any admin-related work. You made everything so easy and smooth. Thank you **Tonja** for organizing the Swedish EBC workshop! Min svenska är mycket bättre nu :-)

Office-mates: **Inga**, thank you for being so inspirational and for the nice chats, you will always be remembered. **Valentina**, by now, you believe in my magical powers. Right! :-) **Adrian Forsythe**, thank you for being so calm, disciplined and organized. I learned some time management skills from you :-) Thank you also for the handy tips related to conda environments and the EBC cluster! **Alice**, thank you for being so approachable :-) It was great having you in our office, though for a very short time.

TAs in other teaching courses: Thank you **Manolis** for the nice chats and help during our Animal Physiology teaching. Thank you **Frauke** for the fun times teaching Plant Physiology on Zoom. Thank you to all other TAs I taught with i.e **Rickard**, **Nikola**, **Imke**, **Julia**, **Fatih**, **Katherina**, **Adrian**, **Andrea** and **Caroline**. It was great teaching with all of you. Thank you, **Peter**, **Afsaneh** and **Banafsheh** for being so helpful during the hectic hours of teaching.

People in EBC: **Merce**, thank you for your passion and enthusiasm for science and academia. **Sifra** and **Charlotta**, it was fun being PhD student

representative in the IOB board with you both, organizing elections and having long discussions every time we met. Thank you **Imke** for sharing the stress of the final months of PhD studies with me :-) **Dolly**, it was great taking courses both with you and from you. Also, thank you for the shoehorn and the book! :-)

Friends: Thank you, both **Caesar** and **Anneli**, for being amazing office-mates, friends and colleagues. Thank you for the fun-chats about science, life, and our gatherings during my PhD. Thank you for arranging the surprise fika after my half time seminar. It meant so much to me :-) **Anne**, thank you for joining our team of cool kids :-) Though you came to SystBio almost in the second half of my PhD but we connected instantly. Thank you for the hangouts and talks in detail about almost every aspect of the PhD studies. Also, thanks for directing me to **Nathalie** for my thesis cover. She did an amazing job :-) **Shadi**, thank you so much for being such a great person and friend. Thank you for the memorable times during the conference in Prague, for the delicious food that you cooked and for all the spontaneous meetups! **David**, thank you for exploring Innsbruck and Maastricht with me and for the nice chit-chats. Thank you for your kind and encouraging words always! **Rachel**, thank you for organizing all the get-togethers and for the fun at PhD symposium, PhD fikas and PhD gasque. **Sarah** and **Mona**, thank you for the fikas and hangouts in the last year of my PhD. **Christina**, thank you for being my family in Sweden. Thank you for your friendship of 13 years and for making everything so special. Meeting and chatting with you have been therapeutic for me. You definitely have a strong role in making my PhD journey smooth :-) Thank you **Steve** and **Huiwen**, for staying in touch with me all these years after our master's program. Thank you for always being a message away, for celebrating my successes with enthusiasm and for travelling from different parts of Europe to Hinxton, Cambridge and Amsterdam to make our meetups possible. **Anum** and **Madiha**, thanks for taking the long trips to Stockholm every now and then just to enjoy a Pakistani breakfast there :-)

Thanks to three important travel grants I received in the final year of my PhD i.e the Early Career Scientist Meeting grant from the FEMS Society, the Helge Ax:son Johnsons Foundation Travel Scholarship and Anna Maria Lundins Travel Scholarship, I was able to attend two leading international conferences in the field.

Family: Thank you, shireen **Dada**, for being an inspiration to me since my childhood. Thank you for your love for science and research. Thank you for being an amazing scientist and a top-notch engineer. I wish I had inherited a bit of your intelligence and smartness. Thank you for buying us all those science encyclopaedias and scientific reasoning books from our early childhood. I still remember the contents of those books. Thank you for your continuous encouragement on pursuing both higher studies and science. **Nan**, thank you for your prayers, love and home remedies for almost everything. I hope that

with this PhD, your dream of calling me a doctor will come true :-) Thank you **Faheem** bhai for the Urdu translation of the summary of my thesis. I loved it :-) I am also grateful to **Brigadier dada**, **nan** and everybody in my in-laws for being very supportive during my PhD studies.

Taj, my husband, you are my rock-solid support. Thank you for taking such good care of our twins throughout my PhD journey. It would not have been possible without you. Thanks for your artistic help with some of the figures :-) and also for working so eagerly on the chemistry of psilocybin. **Ella** and **Mila**, my beautiful twin girls, thank you for putting up with me when I was so busy working on my thesis. Thank you for always cheering me up, brightening up my days and caring for me so much, even though you are still so small. You are the best things that have ever happened to me, and I love you both to the moon and back.

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