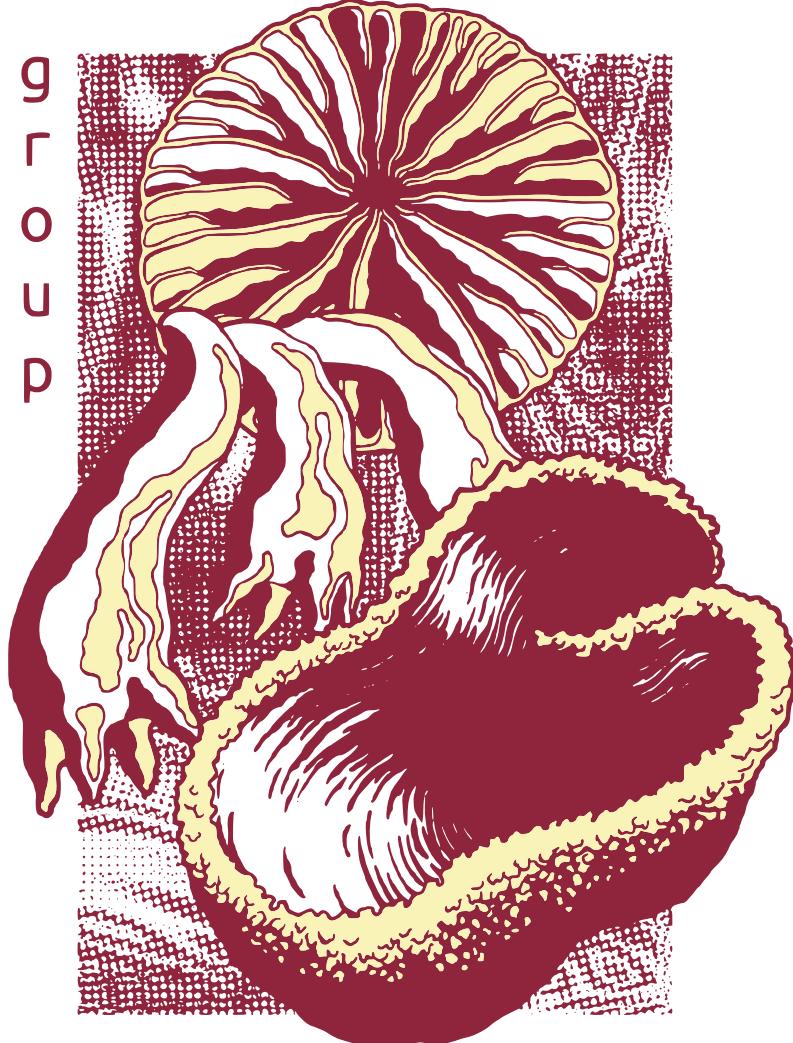


o g
s l o
m y c o l o g y



Case study:
Exploring Archaeorhizomycetes
diversity and distribution with
short- and long-read
metabarcoding

Ella Thoen, post-doc, University of Oslo
ella.thoen@ibv.uio.no



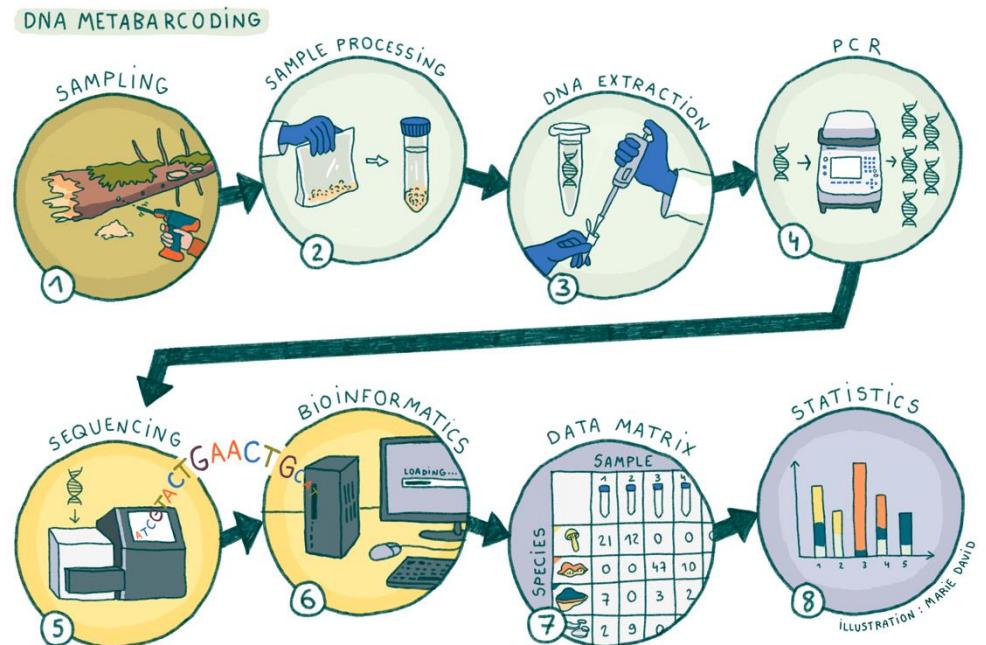
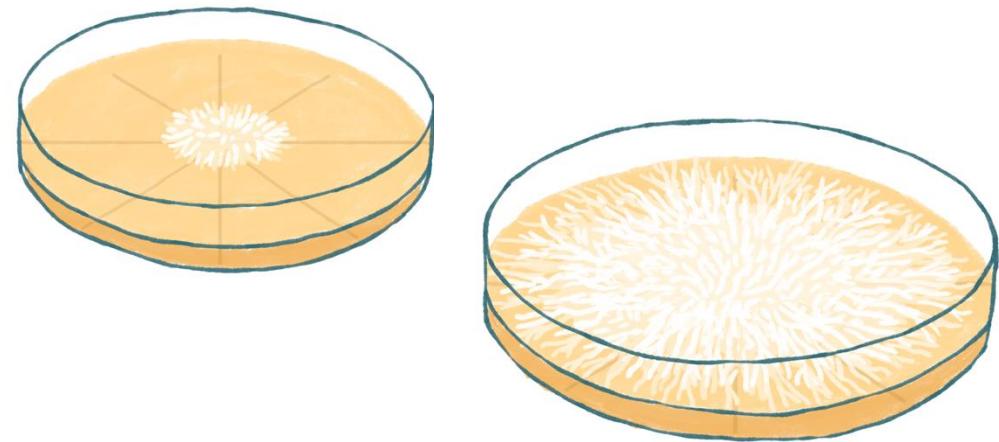
Photo: Ella Thoen

Soils are diverse ecosystems
with numerous **dark taxa**

- Microscopic
- Cryptic lifestyles
- No macroscopic (fruting)-structures
- Typically hard to culture

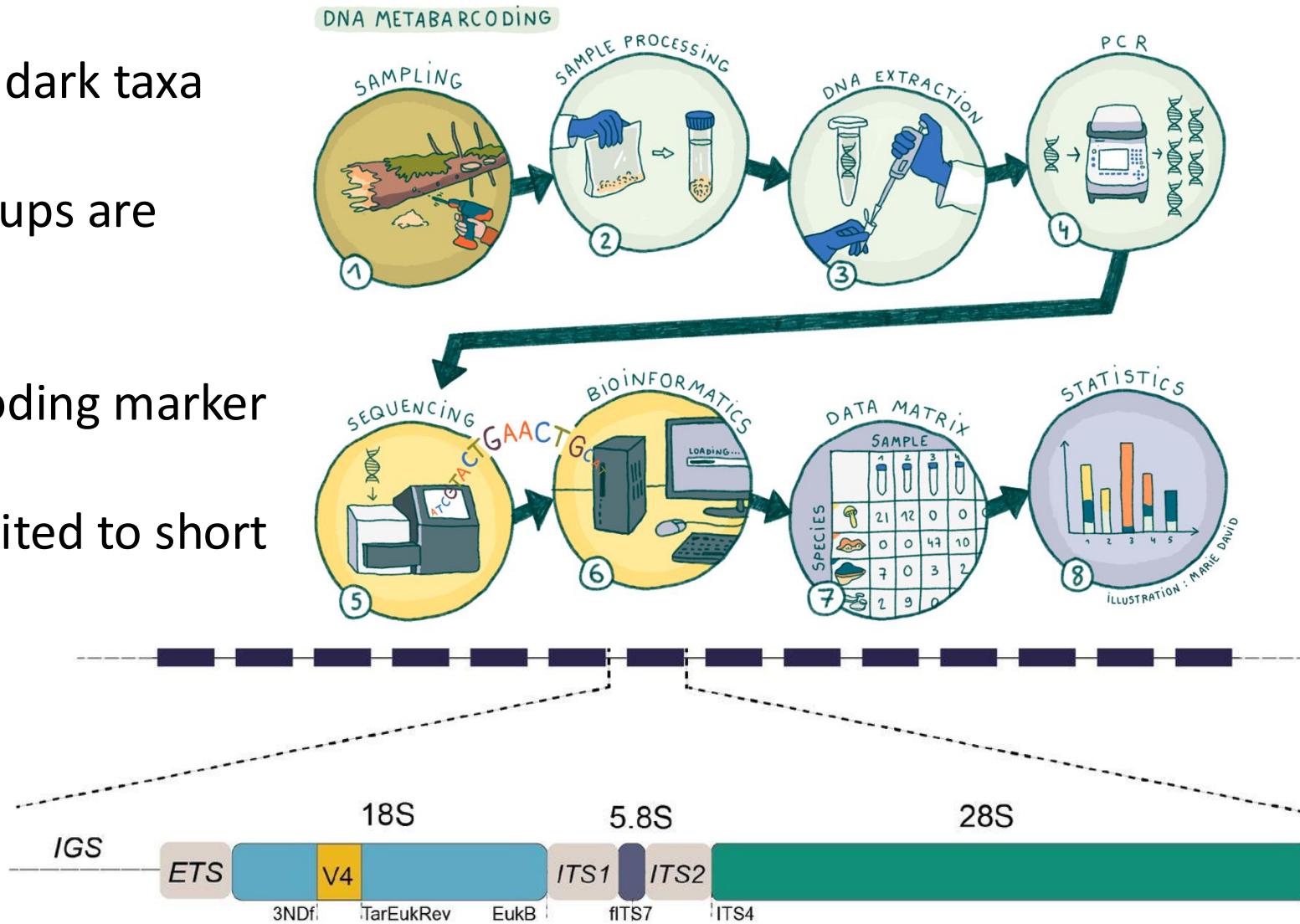
Studying dark fungal taxa

- Culturing
- DNA based methods



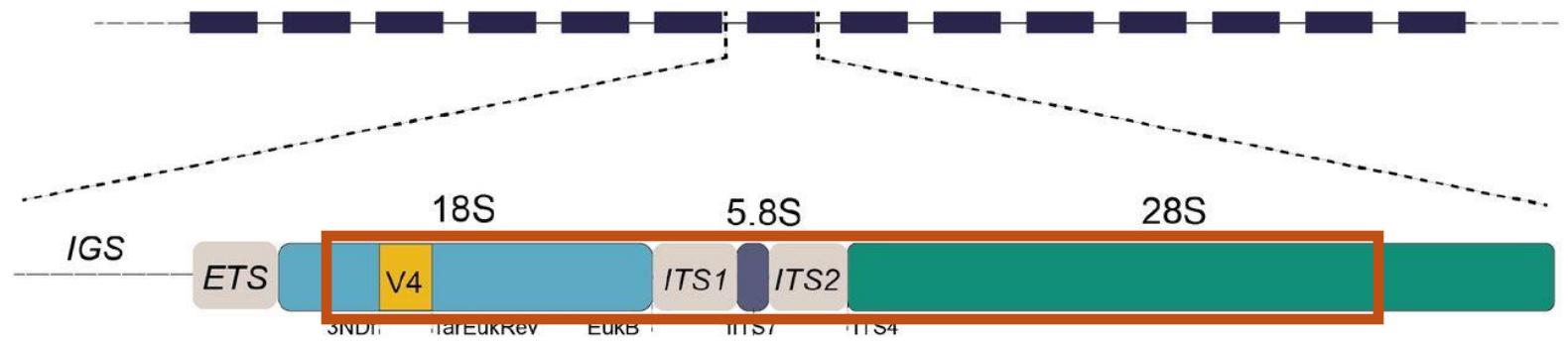
Short-read metabarcoding

- Common method for studying dark taxa
- eDNA is extracted, specific groups are amplified and sequenced
- Dependent on a suitable barcoding marker
- Traditional metabarcoding limited to short reads (< 500 bp)
 - Short markers, limited phylogenetic information



Long-read metabarcoding

- Up to 20 000 bp amplicons and 99.9% accuracy (with PacBio and circular consensus sequences (CCS))
- Longer reads provides better phylogenetic and taxonomic resolution
- Long-read sequencing is a valuable addition to traditional short-read metabarcoding, **especially with dark taxa**



A scanning electron micrograph showing a dense network of thin, light-colored fungal hyphae against a dark background. Some larger, rounded structures, likely conidia or spores, are visible among the hyphae.

Archaeorhizomycetes

10µm
—

Seasonal Dynamics of Previously Unknown Fungal Lineages in Tundra Soils

Christopher W. Schadt,^{1*} Andrew P. Martin,¹ David A. Lipson,²
Steven K. Schmidt^{1†}

The finding that microbial communities are active under snow has changed the estimated global rates of biogeochemical processes beneath seasonal snow packs. We used microbiological and molecular techniques to elucidate the phylogenetic composition of undersnow microbial communities in Colorado, the United States. Here, we show that tundra soil microbial biomass reaches its annual peak under snow, and that fungi account for most of the biomass. Phylogenetic analysis of tundra soil fungi revealed a high diversity of fungi and three novel clades that constitute major new groups of fungi (divergent at the subphylum or class level). An abundance of previously unknown fungi that are active beneath the snow substantially broadens our understanding of both the diversity and biogeochemical functioning of fungi in cold environments.

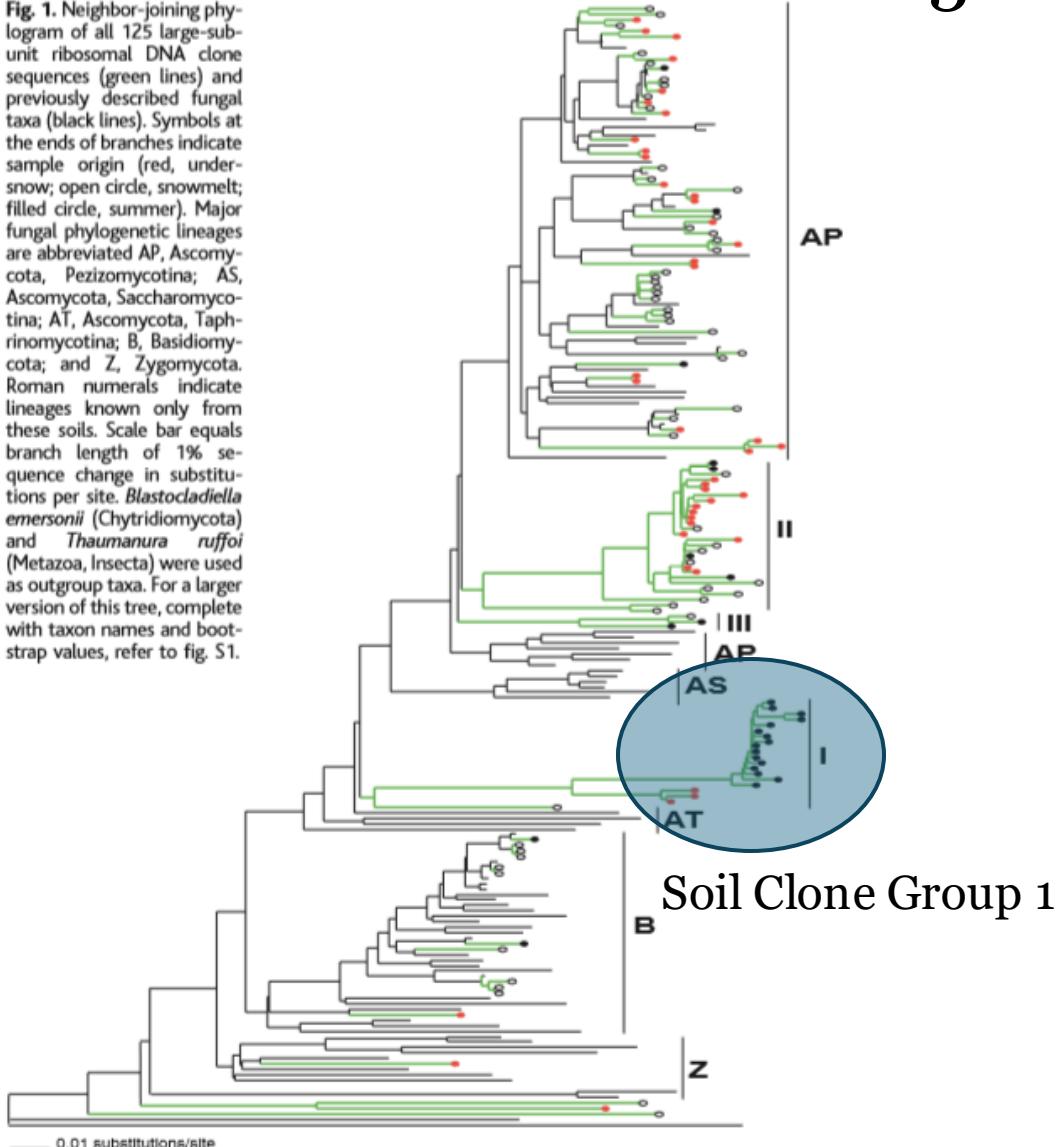
About 40% of the terrestrial environment consists of biomes that are covered by snow for varying lengths of time in the winter (*1*). Soils in these environments contain a large reservoir of organic carbon (*2, 3*). Until recently, it was assumed that soil microorganisms were inactive during the snow-covered period. However, measurements of a high efflux of CO₂ and other greenhouse gases through the snow suggest that microbial populations can be active under the snow (*4–6*).

These results prompted a reevaluation of whether some seasonally snow-covered environments are sinks of atmospheric CO₂ (*7*). In addition, undersnow microbial metabolism is an important biogeochemical sink for nitrogen (*8*), and the subsequent release of microbial nitrogen during snowmelt is a major contributor to high primary productivity during the short growing season in the tundra (*8, 9*). Despite this progress, we know little about the identity or seasonal dynamics of the microbes involved.

We used standard methods (*10*) to estimate microbial biomass in cold soils (*8, 11*). The results show that microbial biomass varies seasonally and reaches maximum annual levels during late winter under the snow in tundra soils (Table 1) (*P* < 0.001). This observation parallels several recent studies that have found peaks in microbial biomass in the late winter (*8, 11, 12*). Most of the

REPORTS

Fig. 1. Neighbor-joining phylogram of all 125 large-subunit ribosomal DNA clone sequences (green lines) and previously described fungal taxa (black lines). Symbols at the ends of branches indicate sample origin (red, undersnow; open circle, snowmelt; filled circle, summer). Major fungal phylogenetic lineages are abbreviated AP, Ascomycota; AS, Ascomycota, Saccharomycotina; AT, Ascomycota, Taphrinomycotina; B, Basidiomycota; and Z, Zygomycota. Roman numerals indicate lineages known only from these soils. Scale bar equals branch length of 1% sequence change in substitutions per site. *Blastocladiella emersonii* (Chytridiomycota) and *Thaumanura rufoi* (Metazoa, Insecta) were used as outgroup taxa. For a larger version of this tree, complete with taxon names and bootstrap values, refer to fig. S1.





Available online at www.sciencedirect.com



Molecular Phylogenetics and Evolution 46 (2008) 635–644

MOLECULAR
PHYLOGENETICS
AND
EVOLUTION

2008

www.elsevier.com/locate/ympev

Widespread occurrence and phylogenetic placement of a soil clone group adds a prominent new branch to the fungal tree of life

T.M. Porter^{a,*}, C.W. Schadt^{b,d,1}, L. Rizvi^c, A.P. Martin^d, S.K. Schmidt^d,
L. Scott-Denton^d, R. Vilgalys^e, J.M. Moncalvo^{a,c}

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Received 30 May 2007; revised 28 September 2007; accepted 6 October 2007

Available online 13 October 2007

Soil Clone
Group 1

Abstract

Fungi are one of the most diverse groups of Eukarya and play essential roles in terrestrial ecosystems as decomposers, pathogens and mutualists. This study unifies disparate reports of unclassified fungal sequences from soils of diverse origins and anchors many of them in a well-supported clade of the Ascomycota equivalent to a subphylum. We refer to this clade as Soil Clone Group I (SCGI). We expand the breadth of environments surveyed and develop a taxon-specific primer to amplify 2.4 kbp rDNA fragments directly from soil. Our results also expand the known range of this group from North America to Europe and Australia. The ancient origin of SCGI implies that it may represent an important transitional form among the basal Ascomycota groups. SCGI is unusual because it currently represents the only major fungal lineage known only from sequence data. This is an important contribution towards building a more complete fungal phylogeny and highlights the need for further work to determine the function and biology of SCGI taxa.

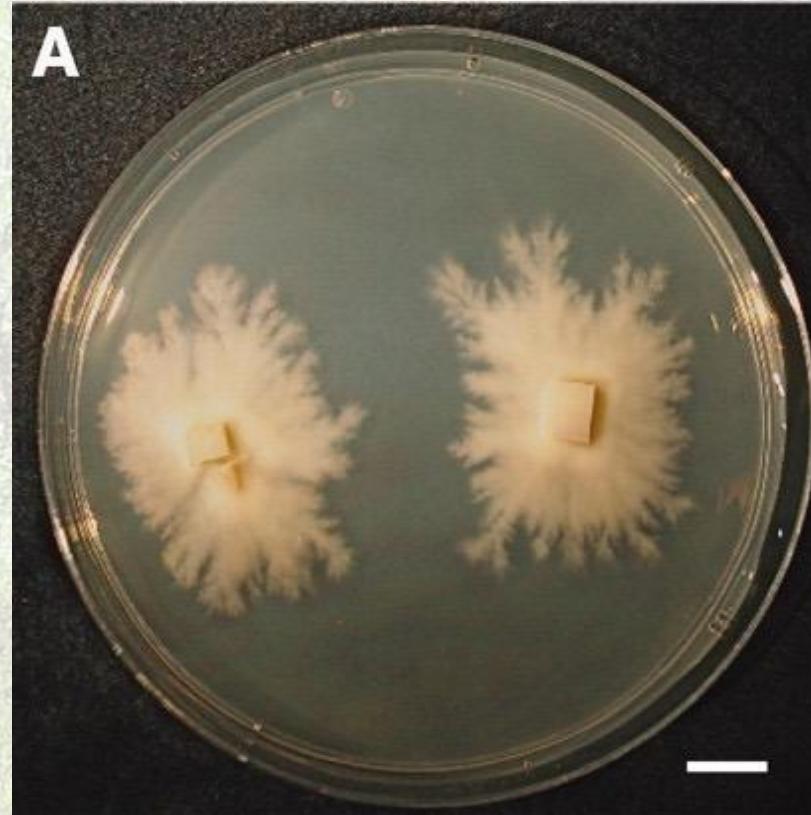
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Keywords: Fungi; Ascomycota; Environmental sampling; Soil; Novel taxa; rDNA

2011



Anna Rosling

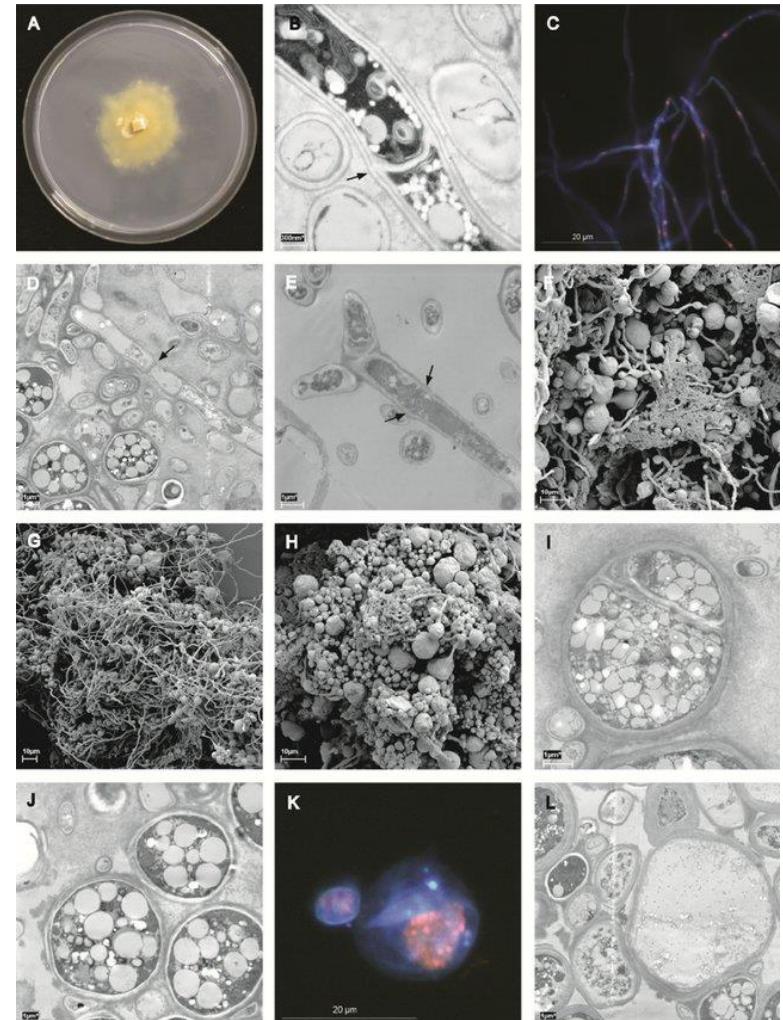


DNA match =
Soil clone group 1

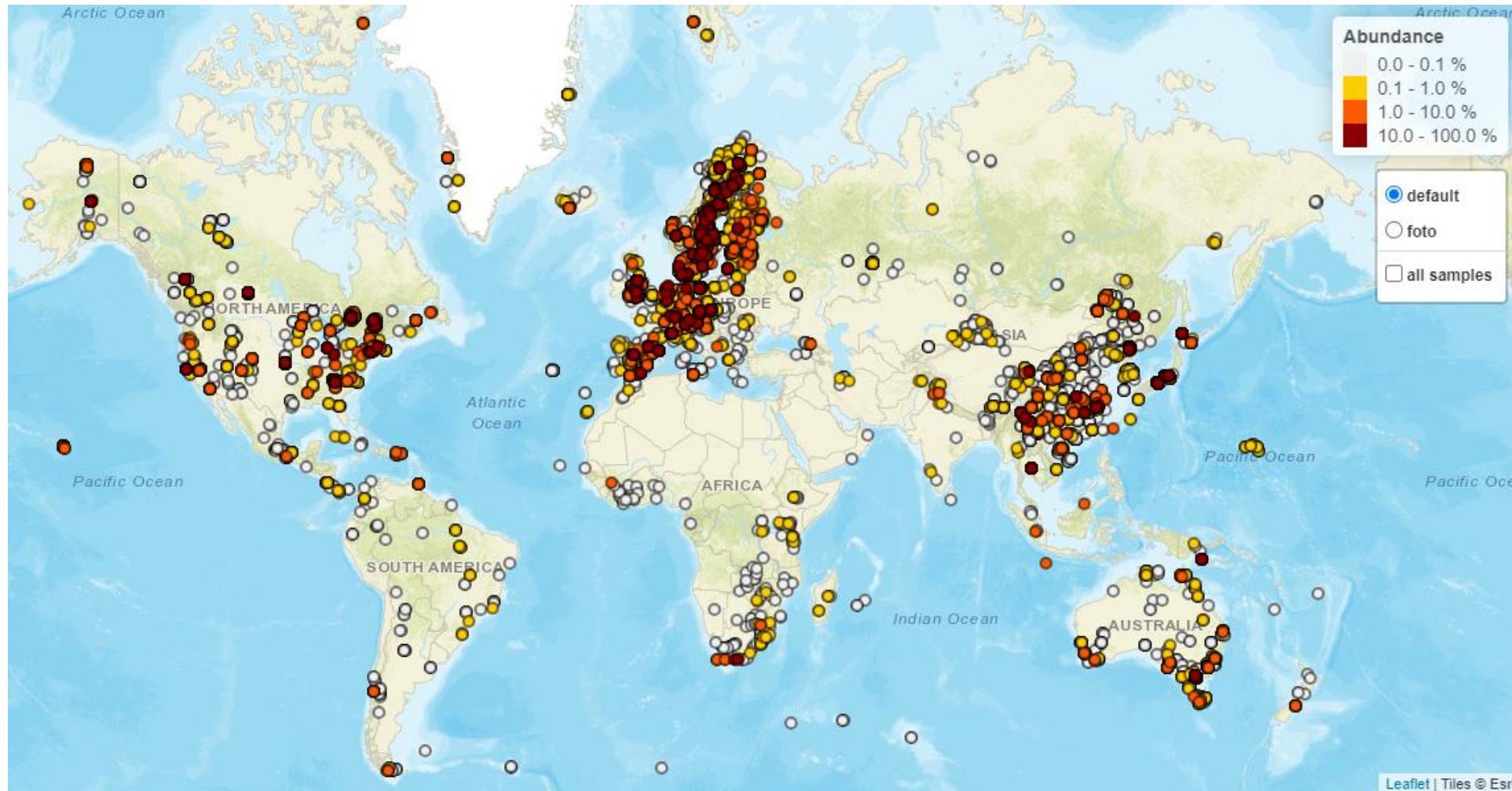
Archaeorhizomycetes

The Archaeorhizomycetes: what do we know?

- Class was formally described in 2011 (Rosling *et al.* 2011) with a culture: *Archaeorhizomyces finlayi* from pine roots
- Species described from culture from pine roots 2014: *A. borealis* (Menkis *et al.* 2014)
- Two new species hypothesis based on long read sequences in boreal forests 2020 (Kahn *et al.* 2020): *A. victor nom. seq* & *A. secuncus nom. seq*
- Two new species from cultures: *A. notokirishmae* & *A. ryukyuensis* (Baba & Hirose 2024)



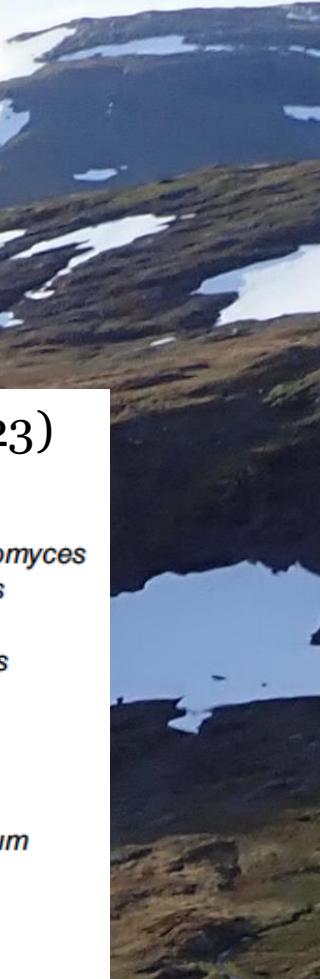
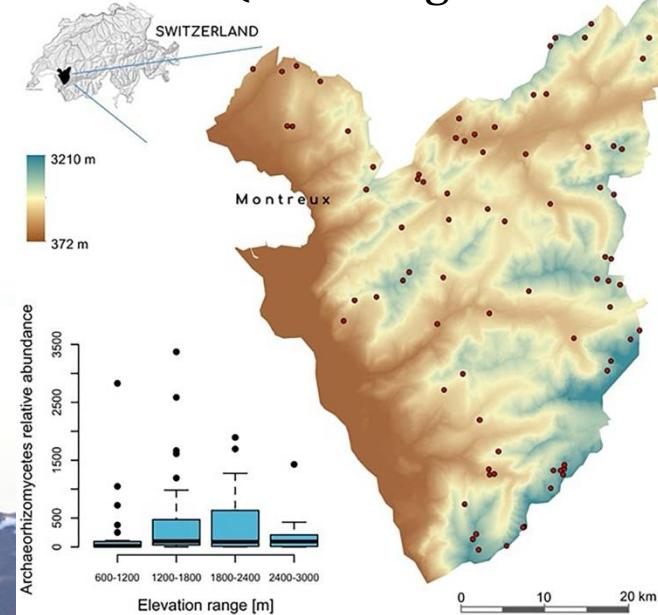
Distribution of Species Hypotheses (1.5%) of Archaeorhizomycetes in the Global Fungi database



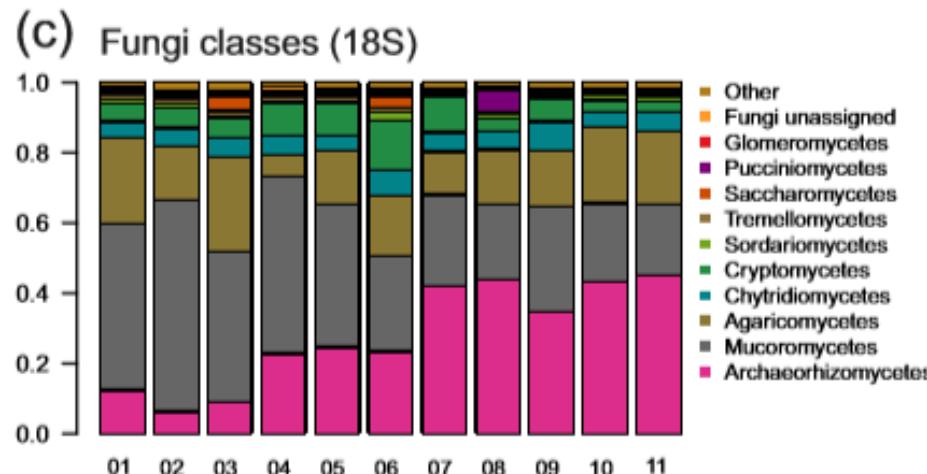
The Archaeorhizomycetes: what do we know?

- Can dominate in some soil samples
- Increases with altitude

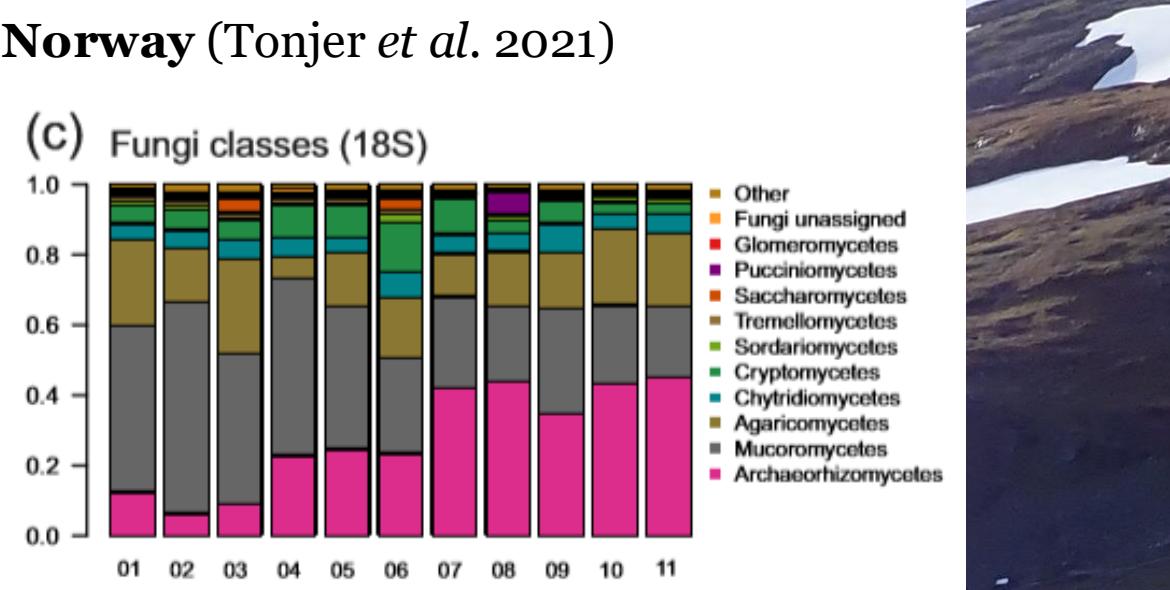
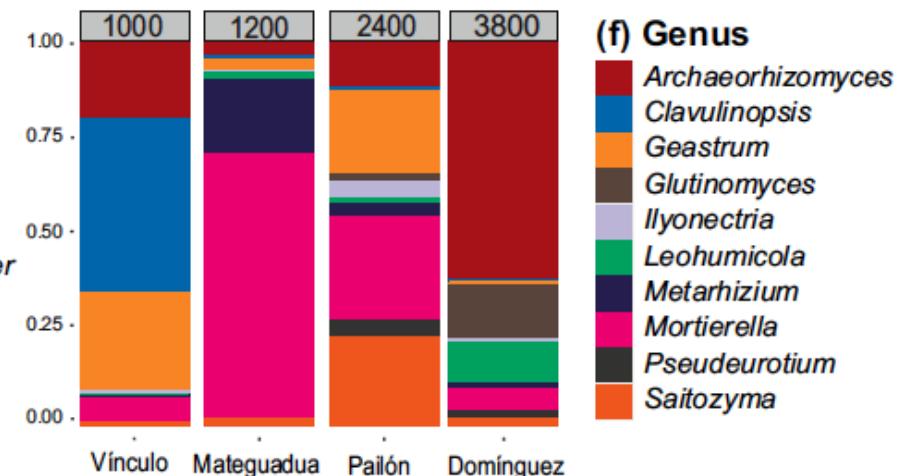
Switzerland (Pinto-Figueroa *et al.* 2019)



Norway (Tonjer *et al.* 2021)



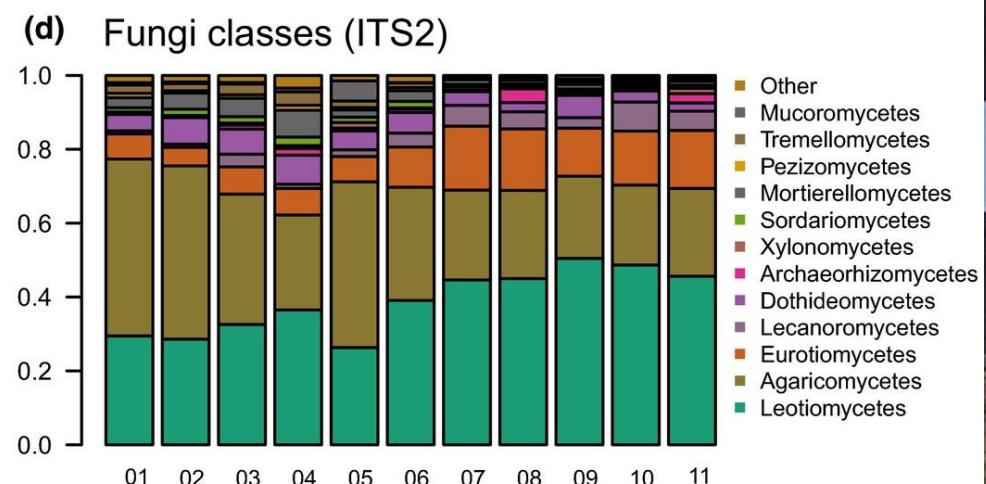
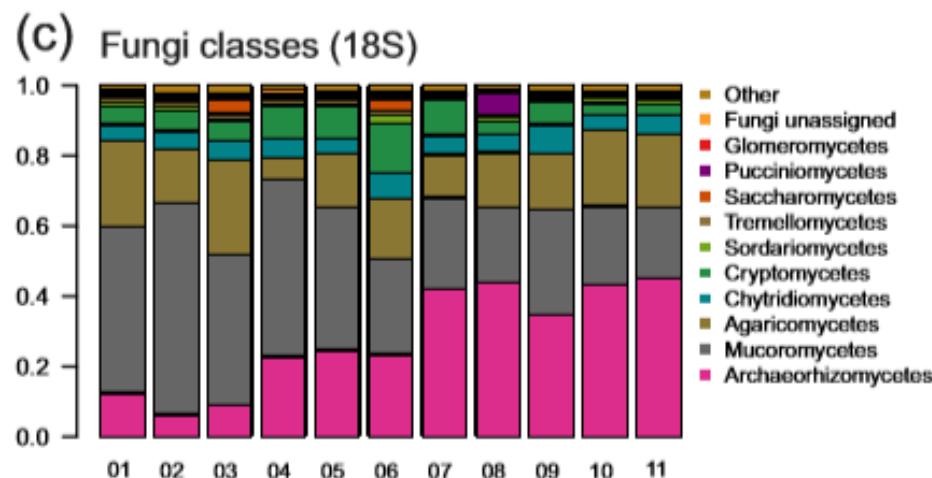
Colombia (Vélez-Martínez *et al.* 2023)



The Archaeorhizomycetes: what do we know?

- Primer bias with the standard ITS2 primers for fungi

Norway (Tonjer *et al.* 2021)



The Archaeorhizomycetes: root associated?

T. Baba and D. Hirose

Fungal Biology 128 (2024) 1939–1953

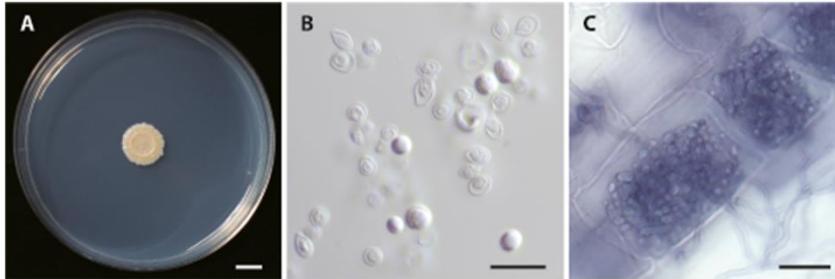


Fig. 3. Morphology of *Archaeorhizomyces notokirishimae* NBRC 116040^T: (A) Colony on MMN after 120 days at 20 °C; (B) yeast cells on MMN; (C) hyphal coils within the hair roots of *Rhododendron kaempferi* stained with trypan blue. Scale bar: 1 cm (A) and 10 µm (B and C).

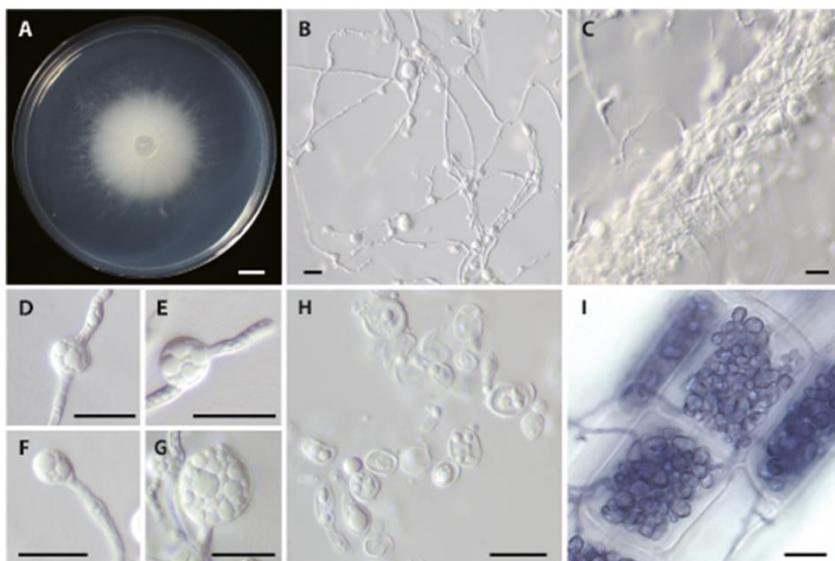


Fig. 4. Morphology of *Archaeorhizomyces ryukyuensis* NBRC 116042^T: (A) Colony on MMN after 120 days at 20 °C; (B-H) microstructure on MMN; (B) mycelial hypha with chlamydospore-like structures; (C) cord-like structure in mycelia; (D, E) intercalary chlamydospore-like structures; (F, G) terminal chlamydospore-like structures; (H) yeast cells; (I) hyphal coils within the hair roots of *Rhododendron kaempferi* stained with trypan blue. Scale bar: 1 cm (A) and 10 µm (B-I).

- Two species isolated from *Pinus sylvestris* roots
(Rosling *et al.* 2011, Menkis *et al.* 2014)
- Two species isolated from ericaceous roots
(Baba and Hirose, 2024)

Archaeorhizomycetes

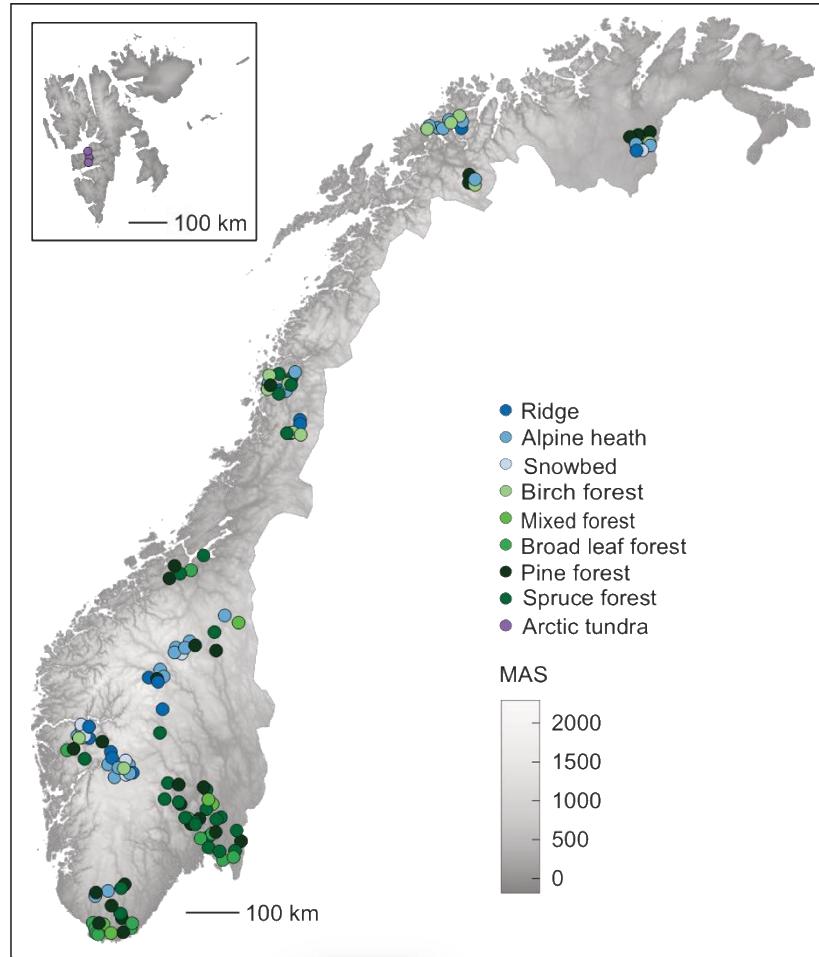
4 described species

Widespread in short-read metabarcoding studies
Unknown/Uncertain ecology/Root associated?

10 μ m



Mapping and describing the diversity of Archaeorhizomycetes in Norway (and Sweden)



Aims

- Plant/vegetation associations? Substrate preferences?
- Other environmental drivers?
- Relative overall abundance compared to other fungal groups?
- Where are they most (proportional) abundant?
- How many taxa can we find?



UNIVERSITY
OF OSLO

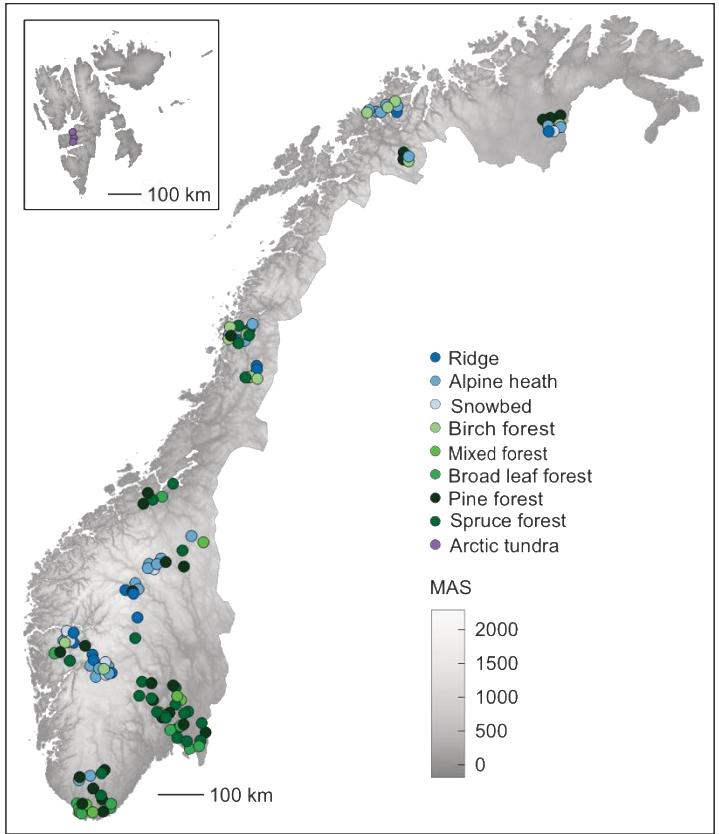


UPPSALA UNIVERSITY
SWEDEN



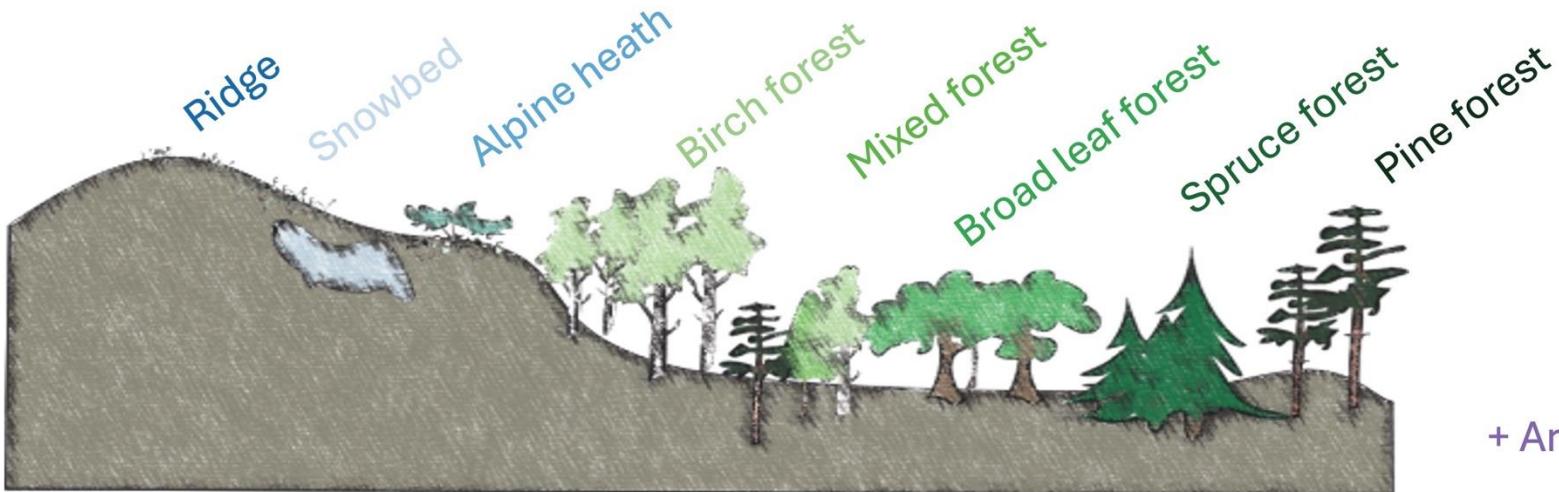
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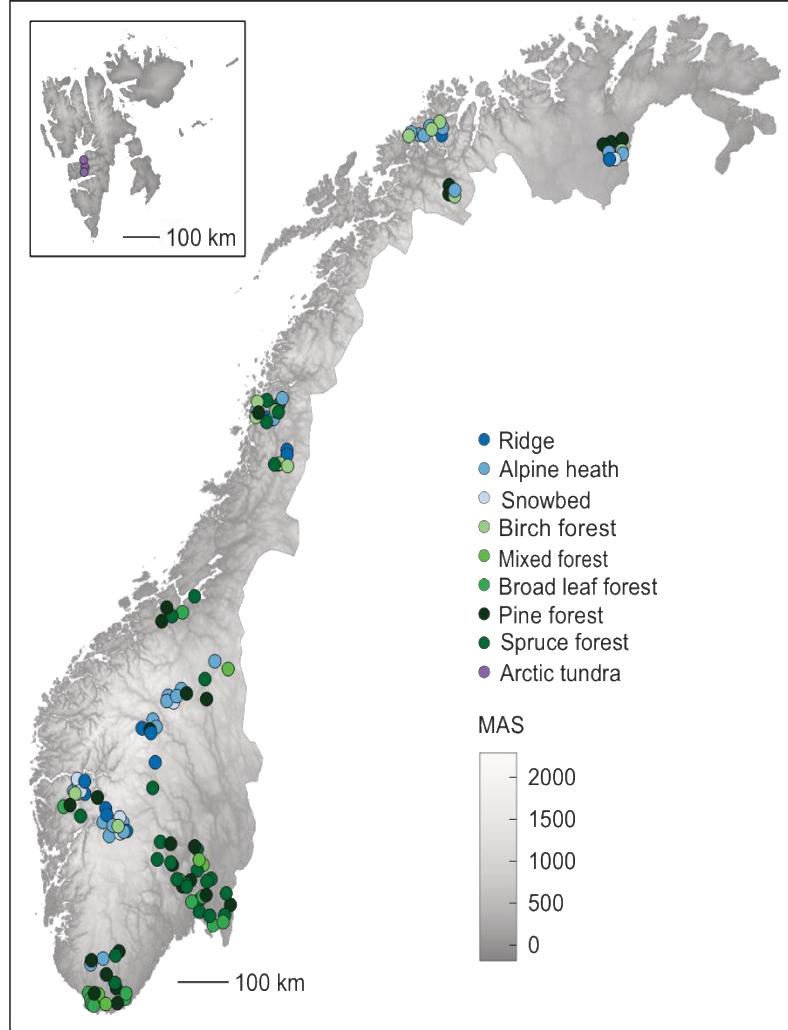
Study design

- 133 sites (forests and alpine habitats + Arctic), 4x4 m
- 3 sample types (soil, litter, plant rots)
- 9 vegetation types

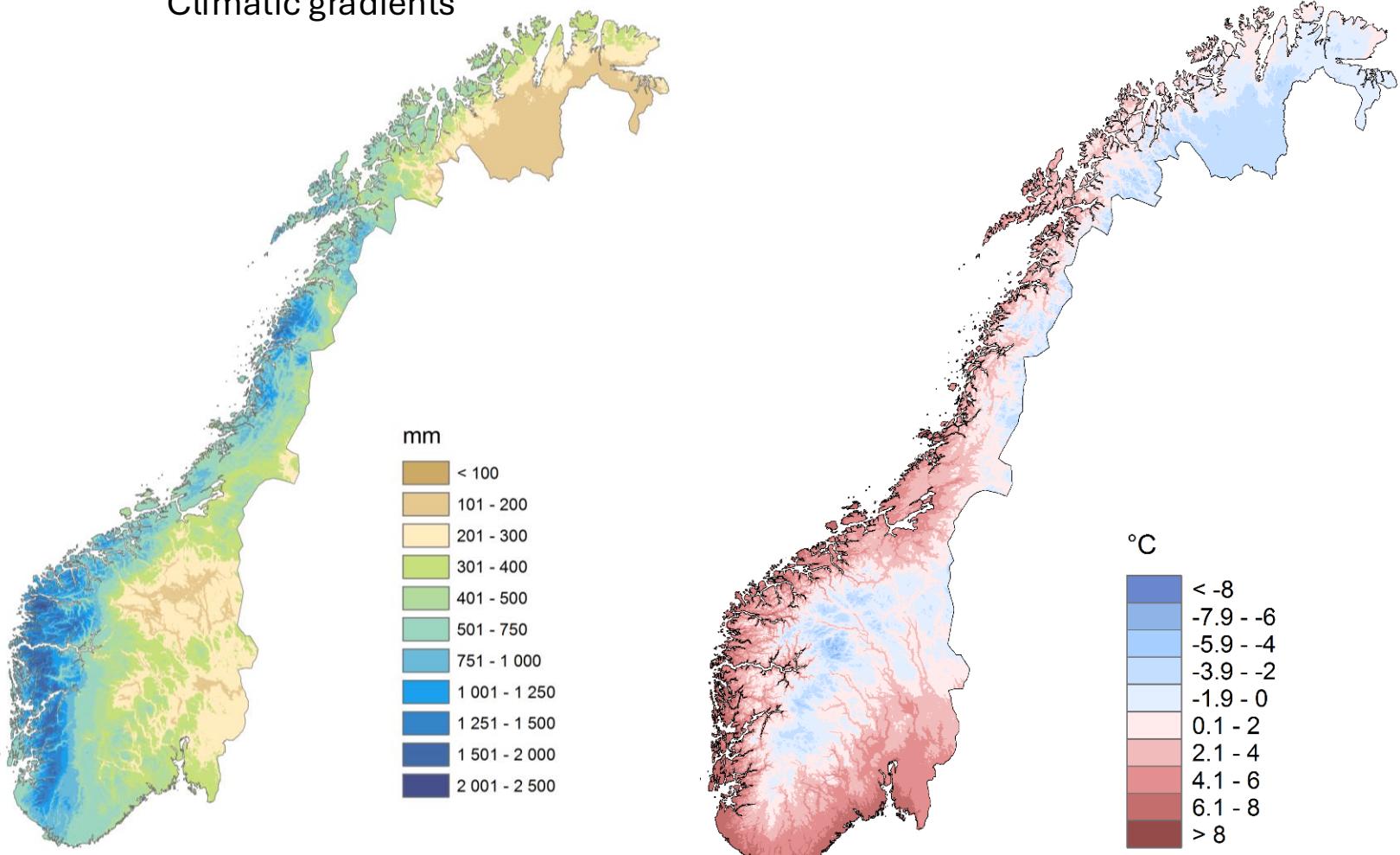


+ Arctic tundra (Svalbard)





Climatic gradients



Field work



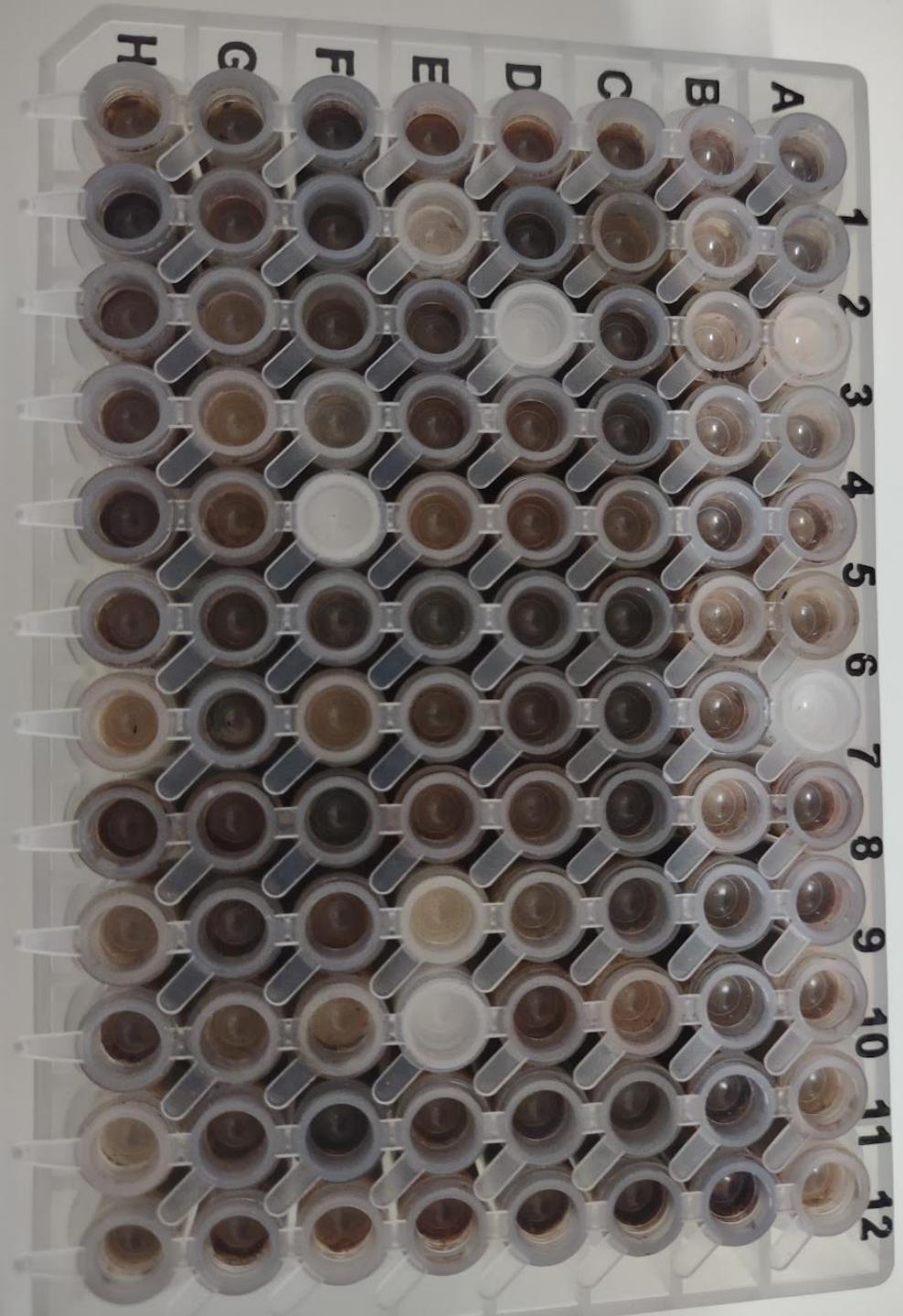








Nutrient gradients

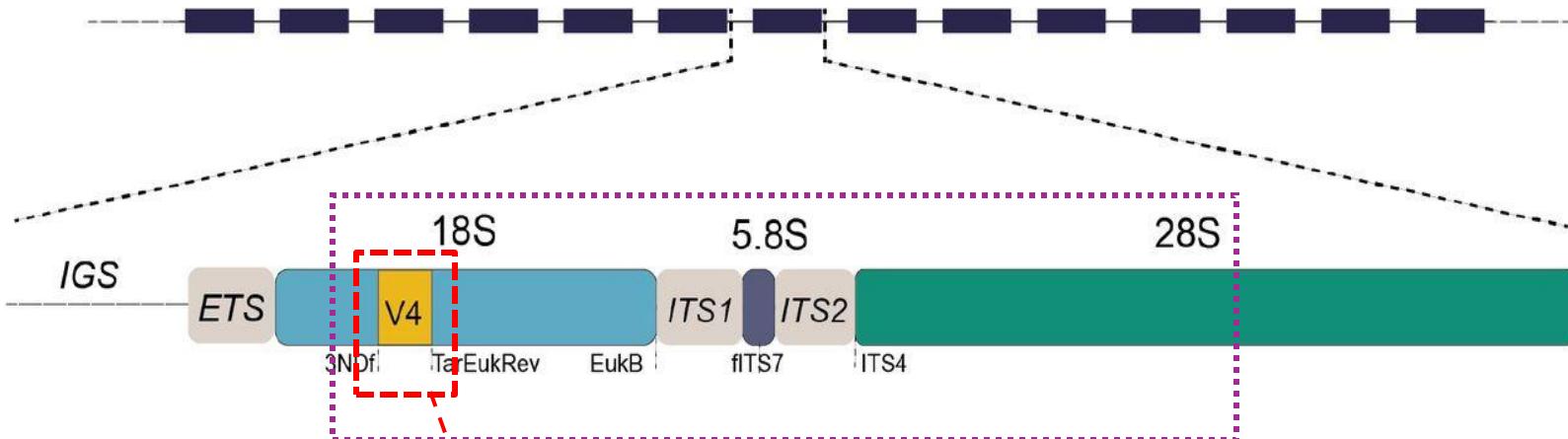


Lab work

- $133 * 3 = 399$ samples
- Samples processed, homogenized, freeze-dried and pulverized
- Extracted in five plates using E.Z.N.A. soil kit



Short- and long-read metabarcoding



Short-read metabarcoding of the V4
region (Illumina miSeq)
~380 bp

→ DADA2 + LULU

Long-read metabarcoding of the
ITS-28S region (PacBio Sequel II)
~2500-3500 bp

18S- → DADA2 + 99% clustering in
VSEARCH +LULU



Primer-design by
Veera Tuovinen
Nogerius, Uppsala
University



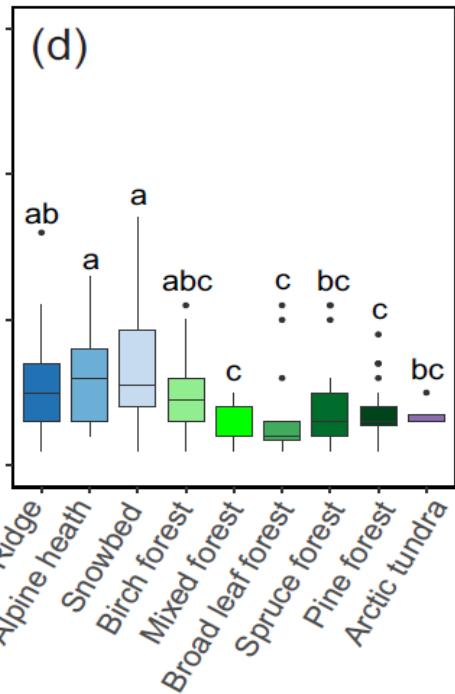
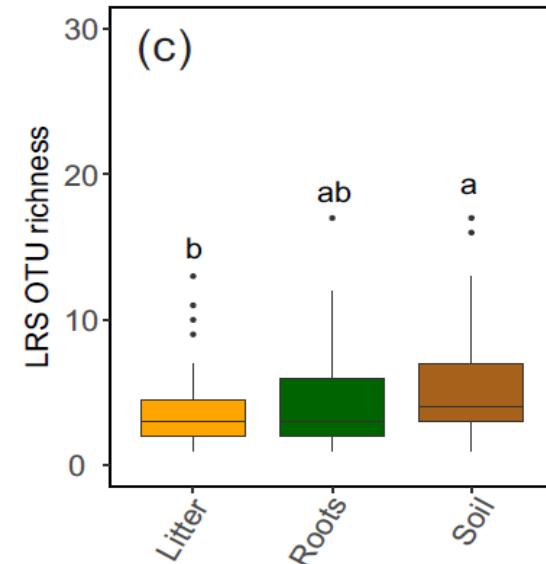
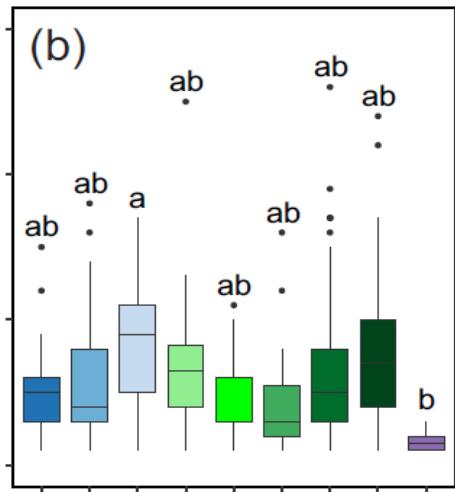
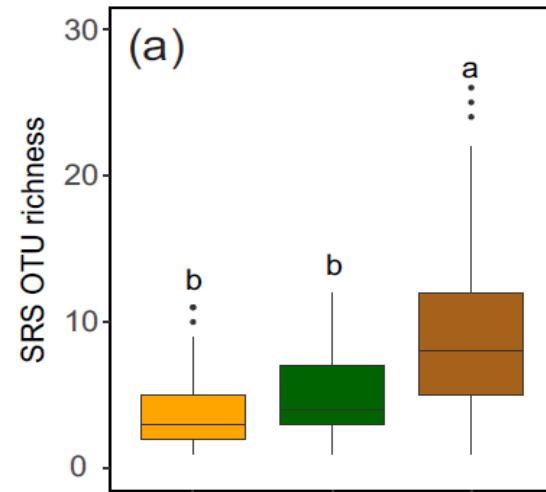
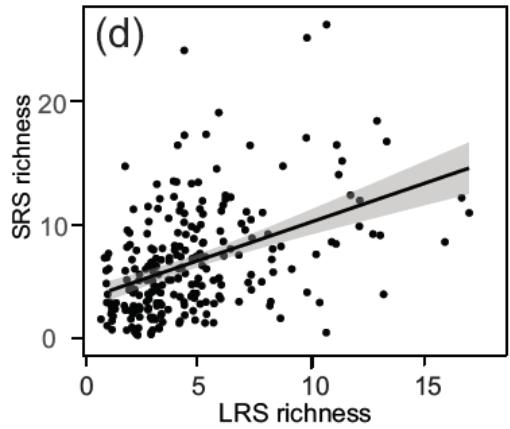
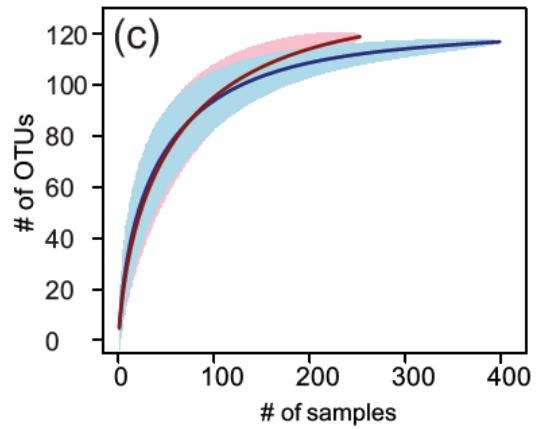
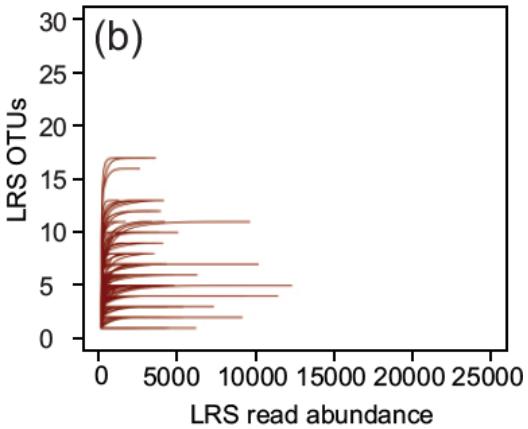
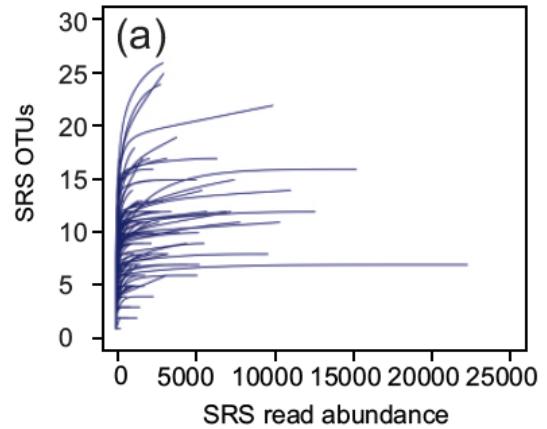
1) Illumina short-read metabarcoding using 18S V4:

- 23,593,549 demultiplexed eukaryotic reads
- Removed plants and Metazoa, duplicate samples, mocks: 4,360,856
- Fungi only: 1,660,554
- Archaeorhizomycetes only: 353,672
- 115 Archaeorhizomycetes OTUs in 353 samples (out of 393)

2) PacBio Hifi long-read sequencing of SSU-ITS-LSU

- 1,538,876 HiFi reads
- After denoising with DADA2: 2,670 ASVs (DADA2), 829,266 reads
- Removed 618 chimeric reads, clustered at 99% and used LULU to correct for oversplitting
- 120 Archaeorhizomycetes OTUs and 790,867 reads in 259 samples (out of 393 (353))

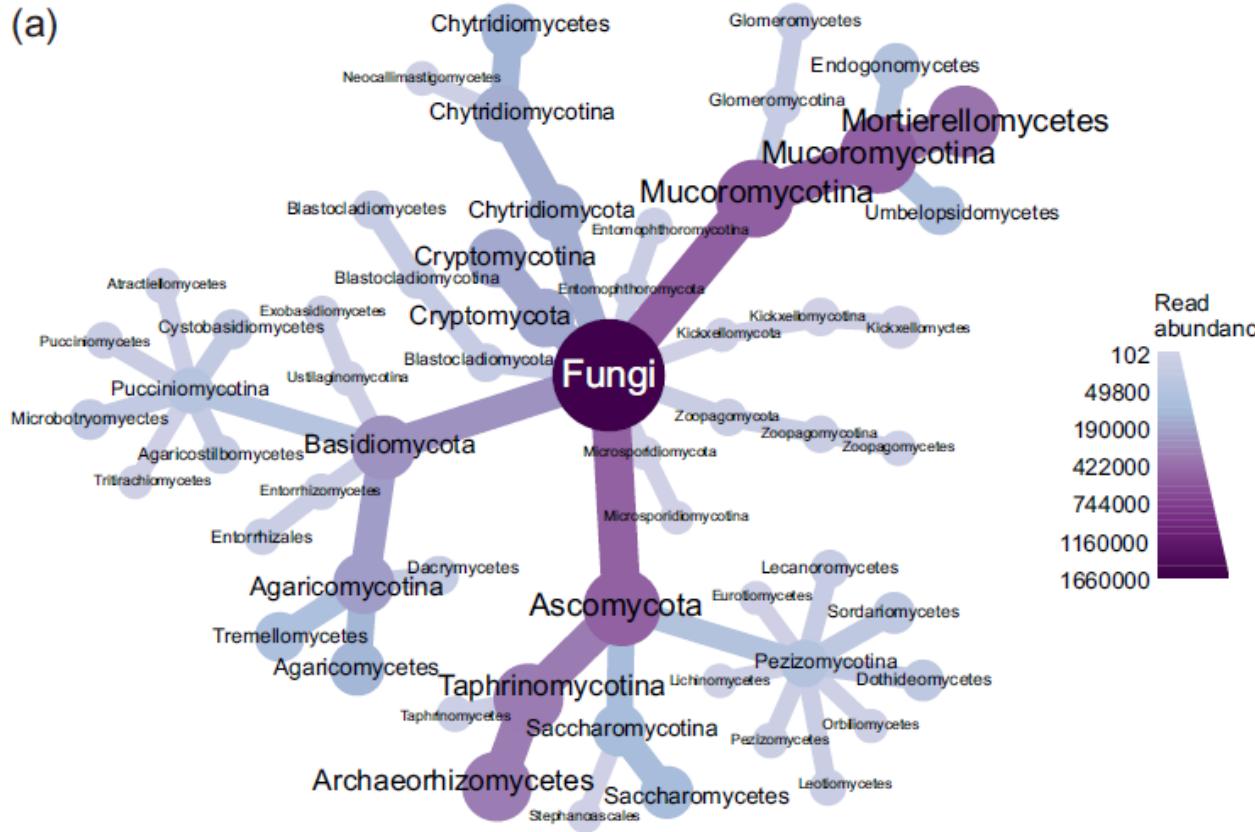
Short-read versus long-read sequence data



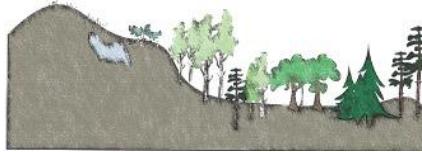
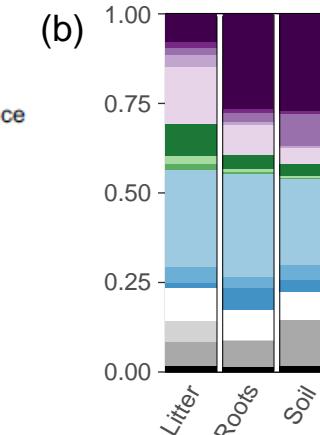
Short read sequences (SRS): 115 OTUs
Long read sequences (LRS): 120 OTUs

Short-read sequence data

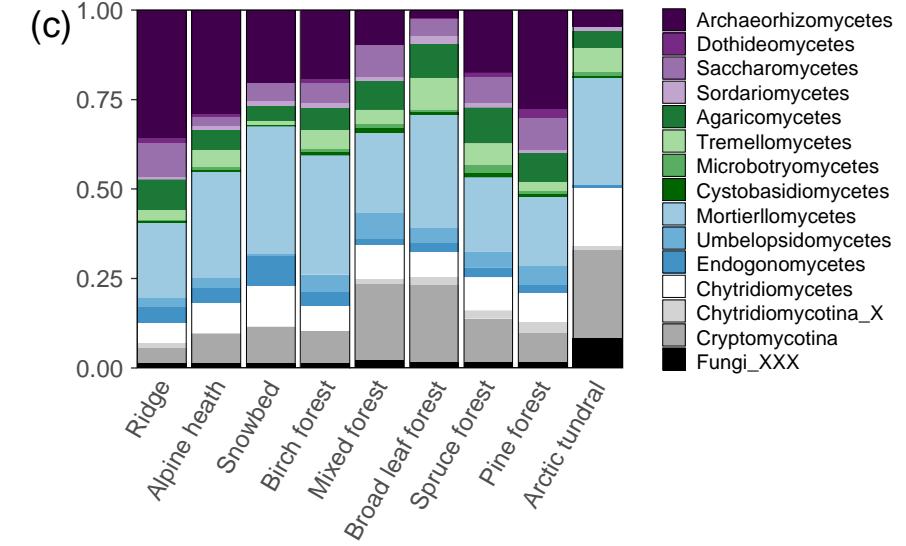
(a)



(b)



(c)



- Second most common fungal class in Norway!
- 0 - 78% of microeukaryotic sequence reads per sample (mean 8%)
- 0 – 88% of fungal sequence reads per sample (mean 19%)

Short-read sequence data



Nutrient poor ridge and heath (alpine)

Nutrient poor forest with
ericaceous understory

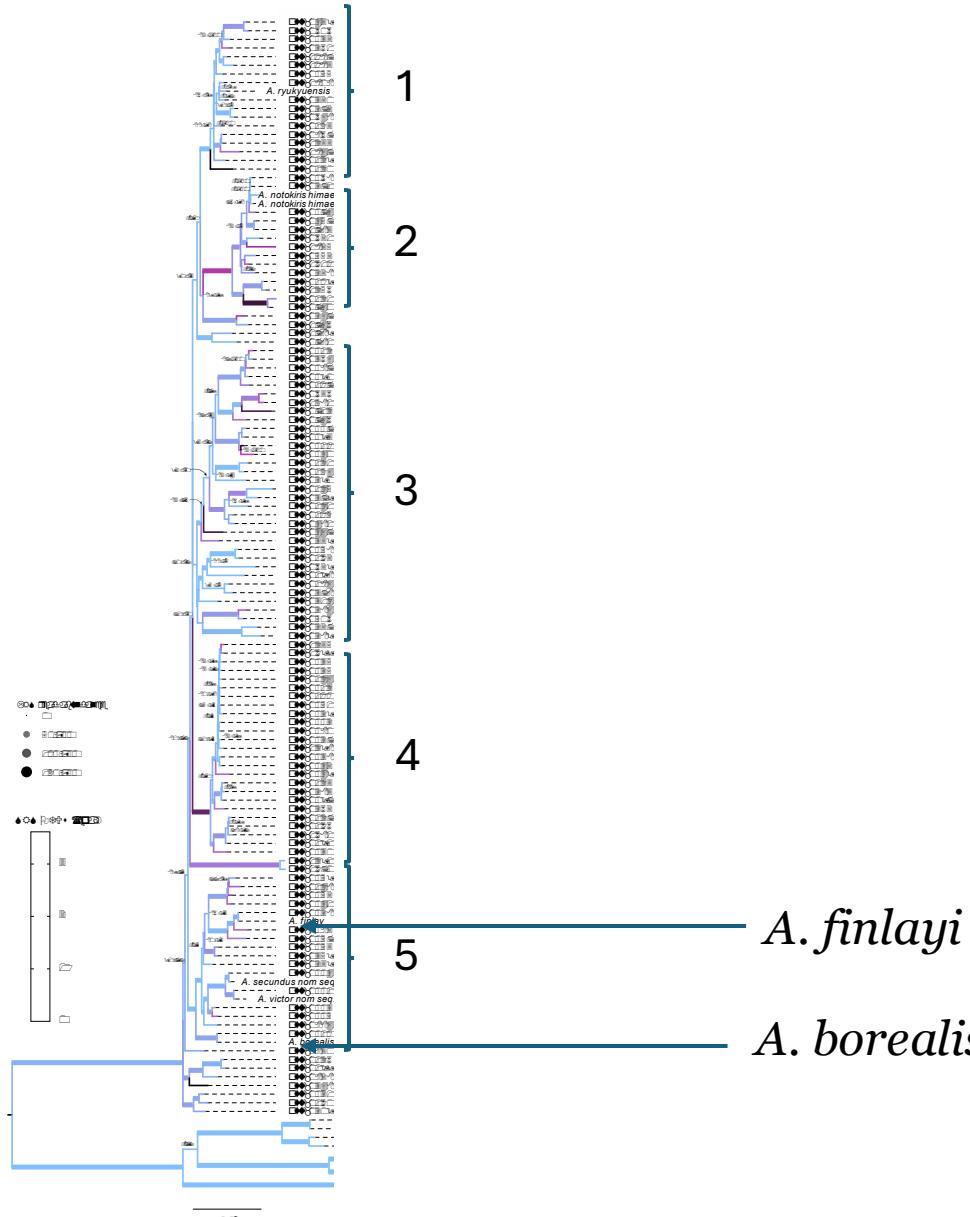
Nutrient rich, moist
broad leaf forest

Beech forest (planted)



Realtive read abundance (short read metabarcoding)

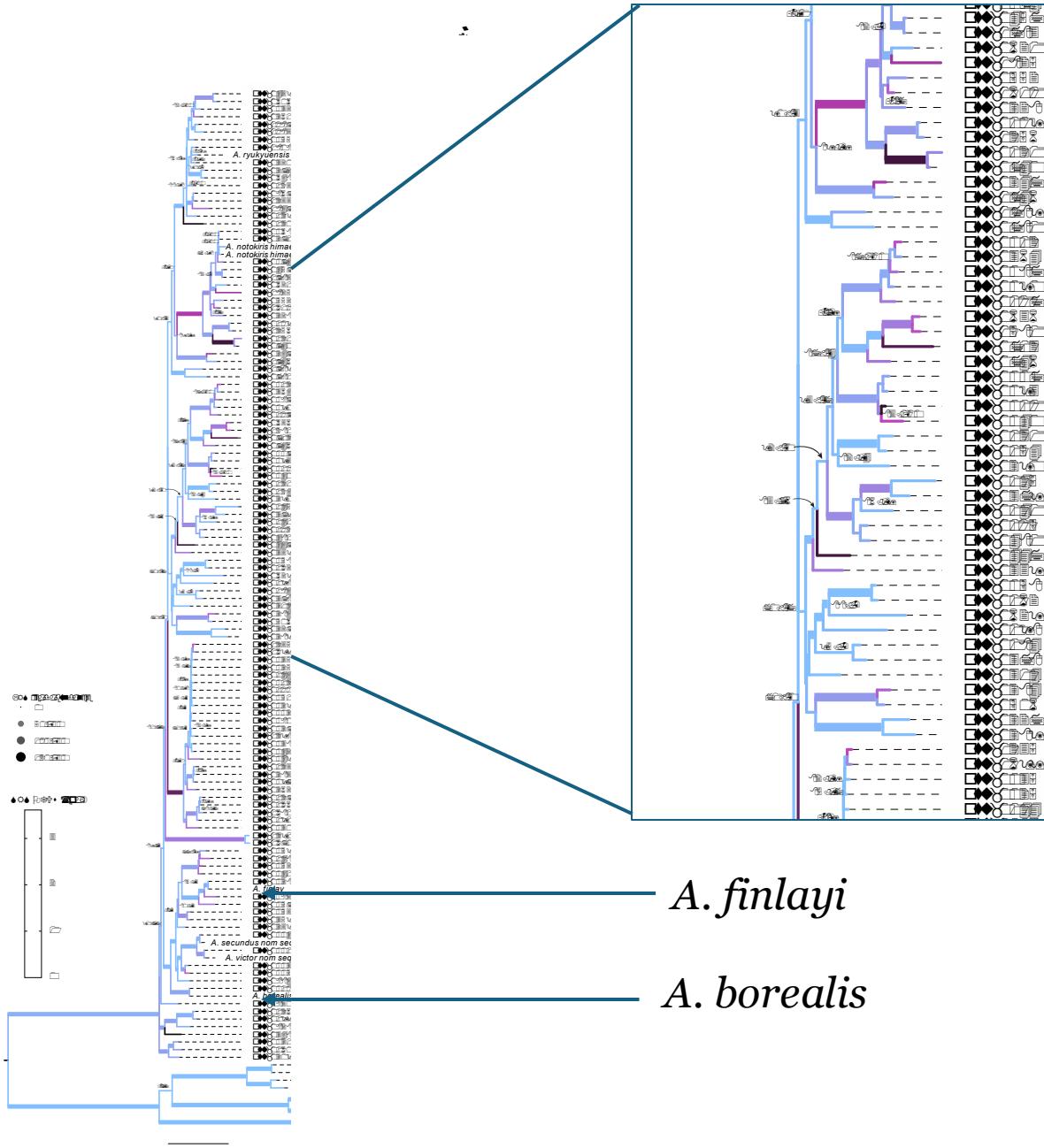
Long-read sequence data



Phylogenetic analyses

- Alignment of 120 LRS OTUs, outlier and *A. finalyi* and *A. borealis* from genomes ('linsi' algorithm in MAFFT v7.505)
- Phylogenetic tree constructed with IQ-TREE (model:GTR+Fxxx, support values with SH-like aLRT test and UFBoot x 1000 replicates)
- **Five major clades**

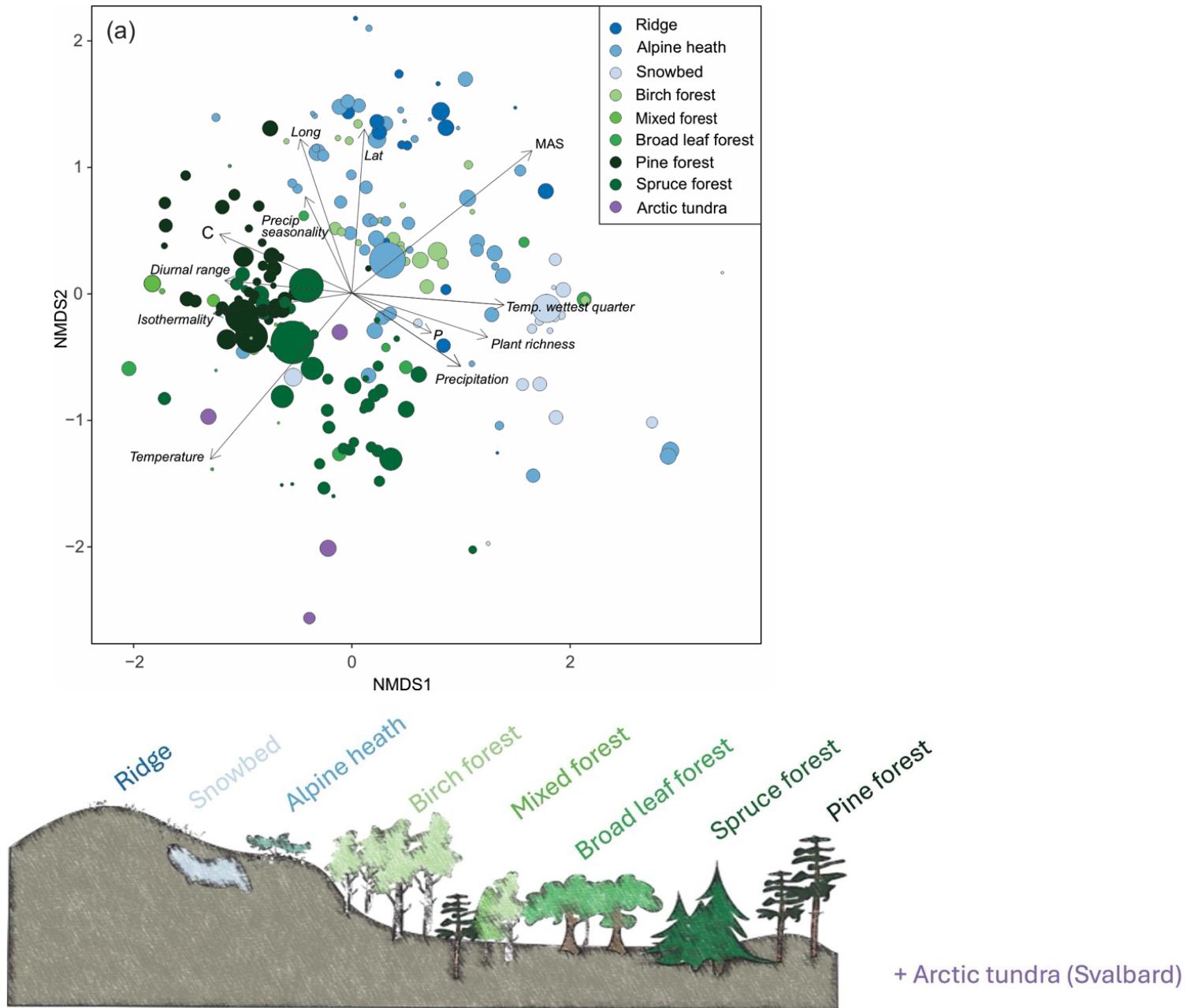




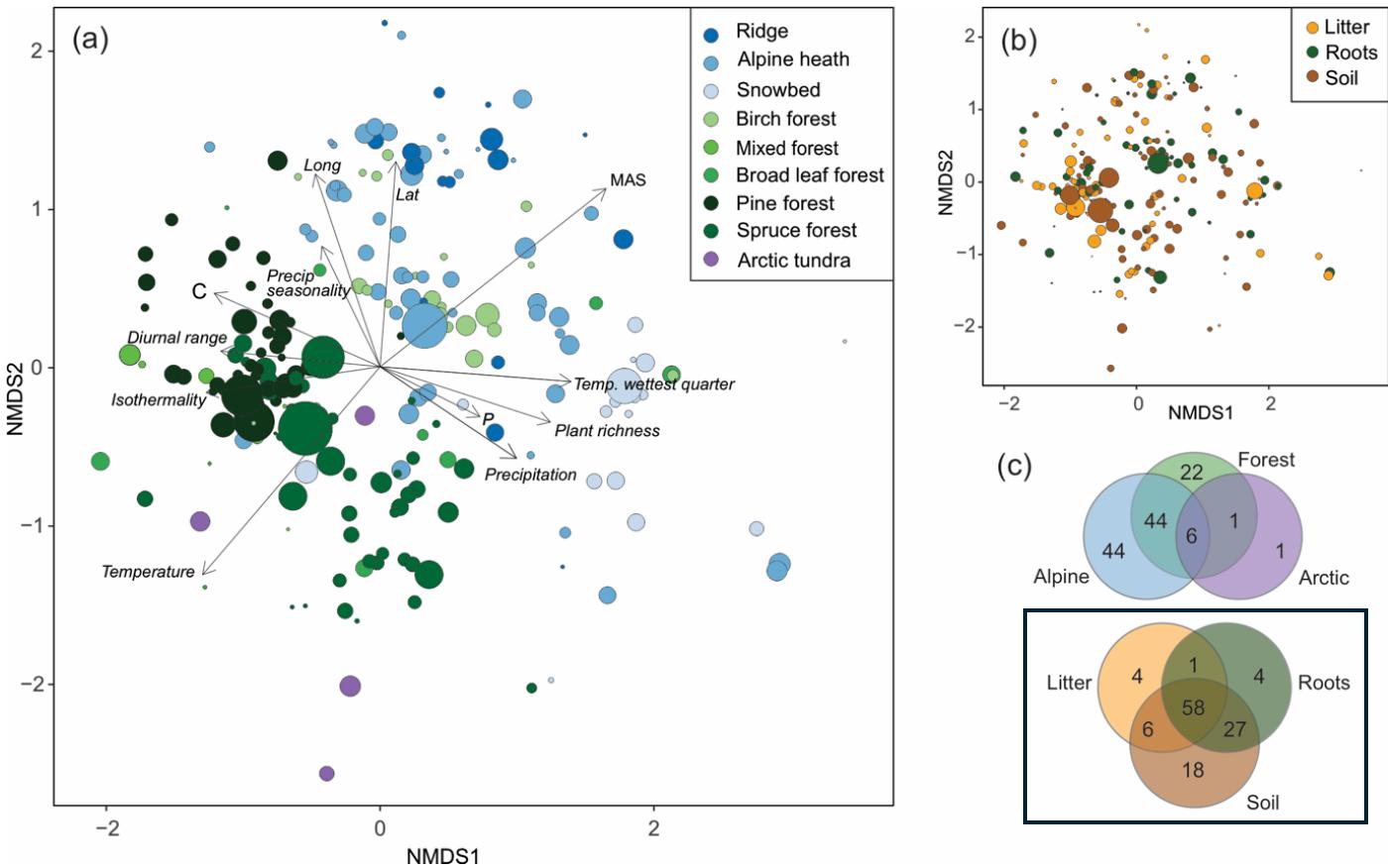
Evolutionary Placement Algorithm (EPA)

- SRS OTUs mapped onto the tree using EPA in RAxML v.8.2.12
- Query sequences are placed on a reference tree individually by a searching algorithm that tries to find the branch with the best likelihood for the query one at the time

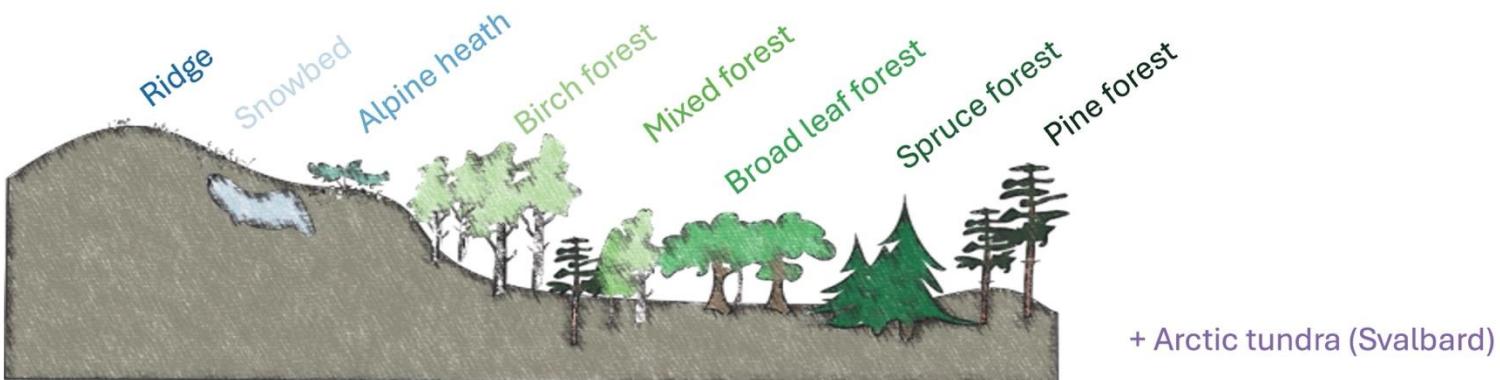
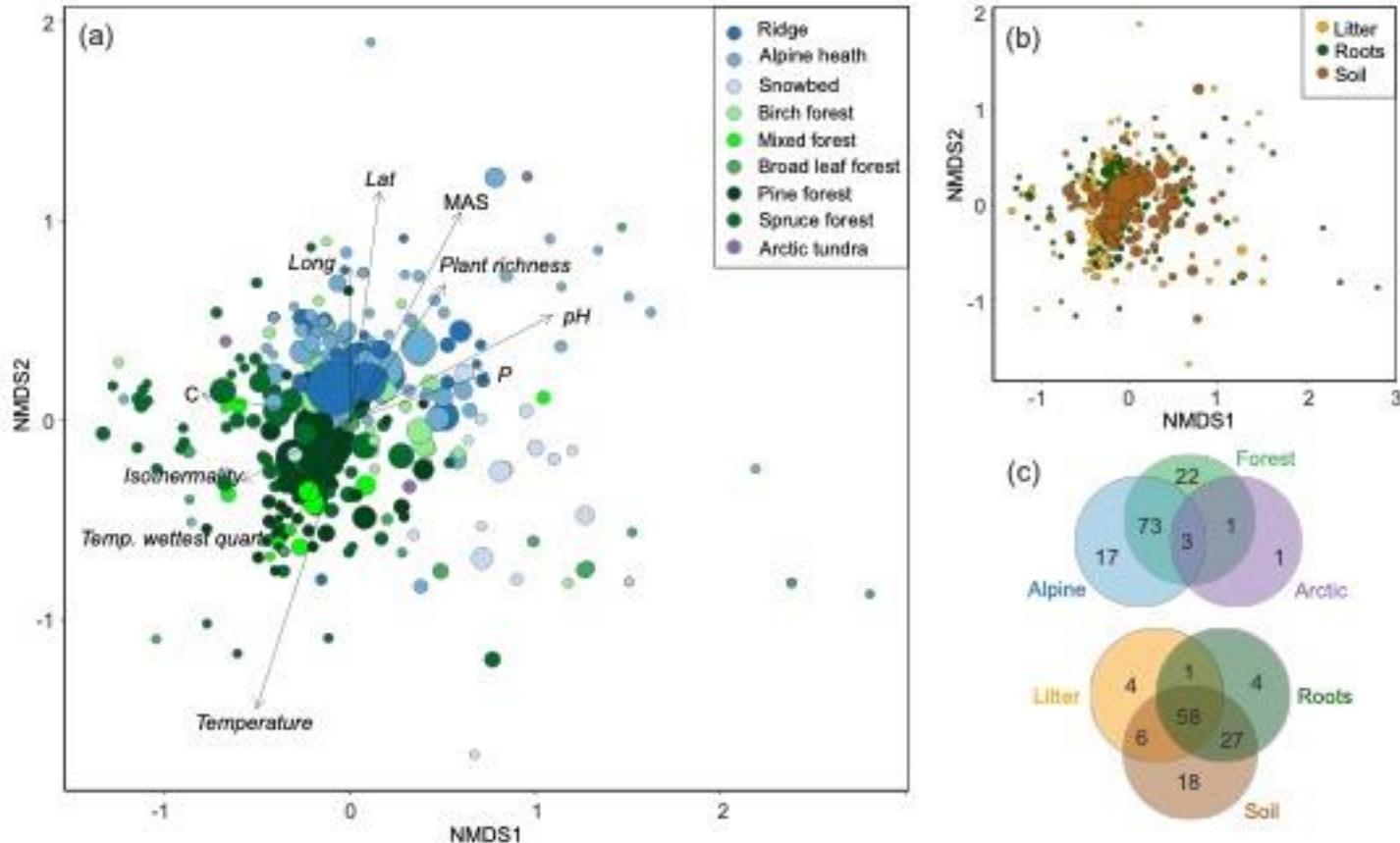
Long-read sequence data



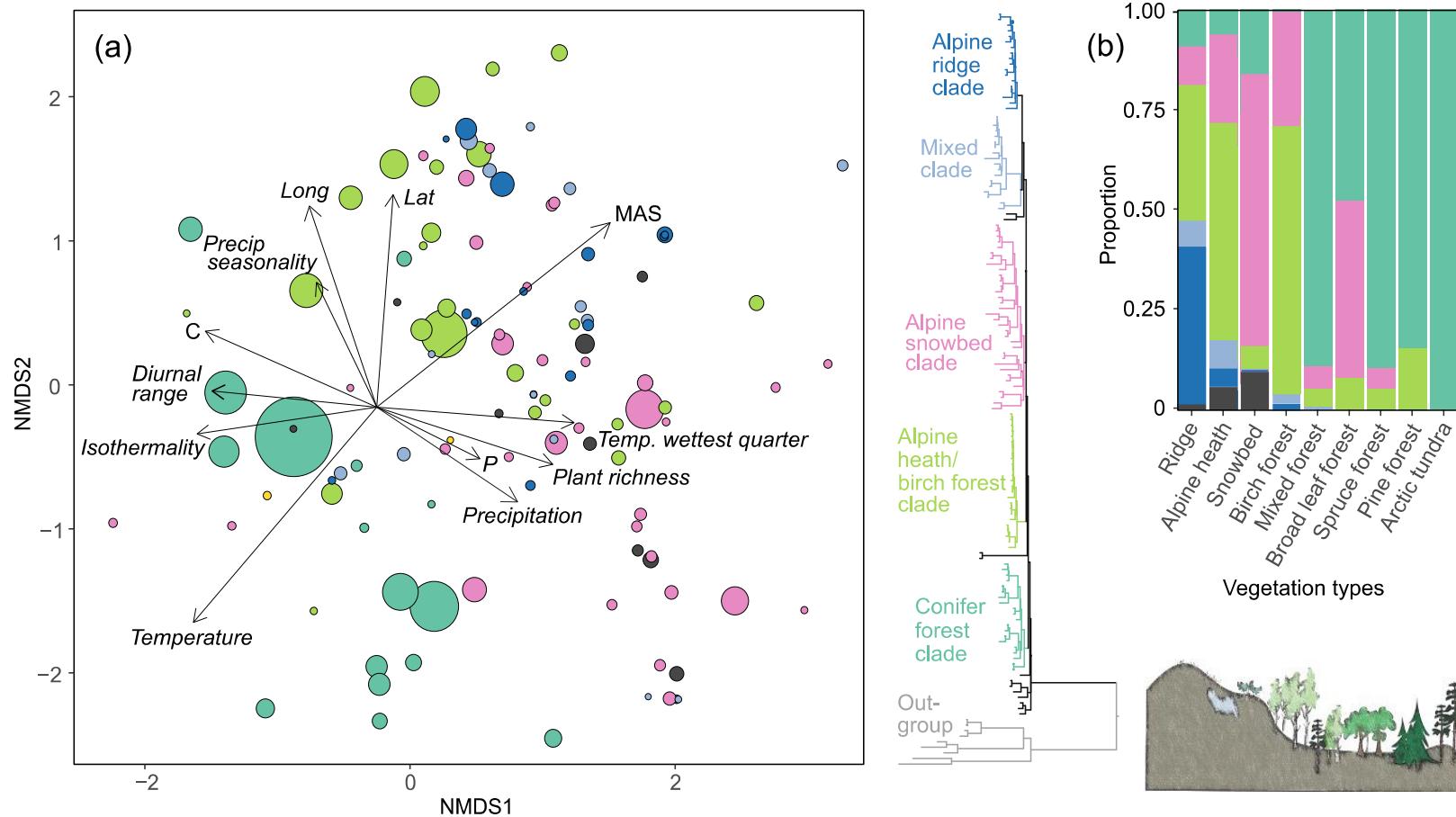
Long-read sequence data



Short-read sequence data

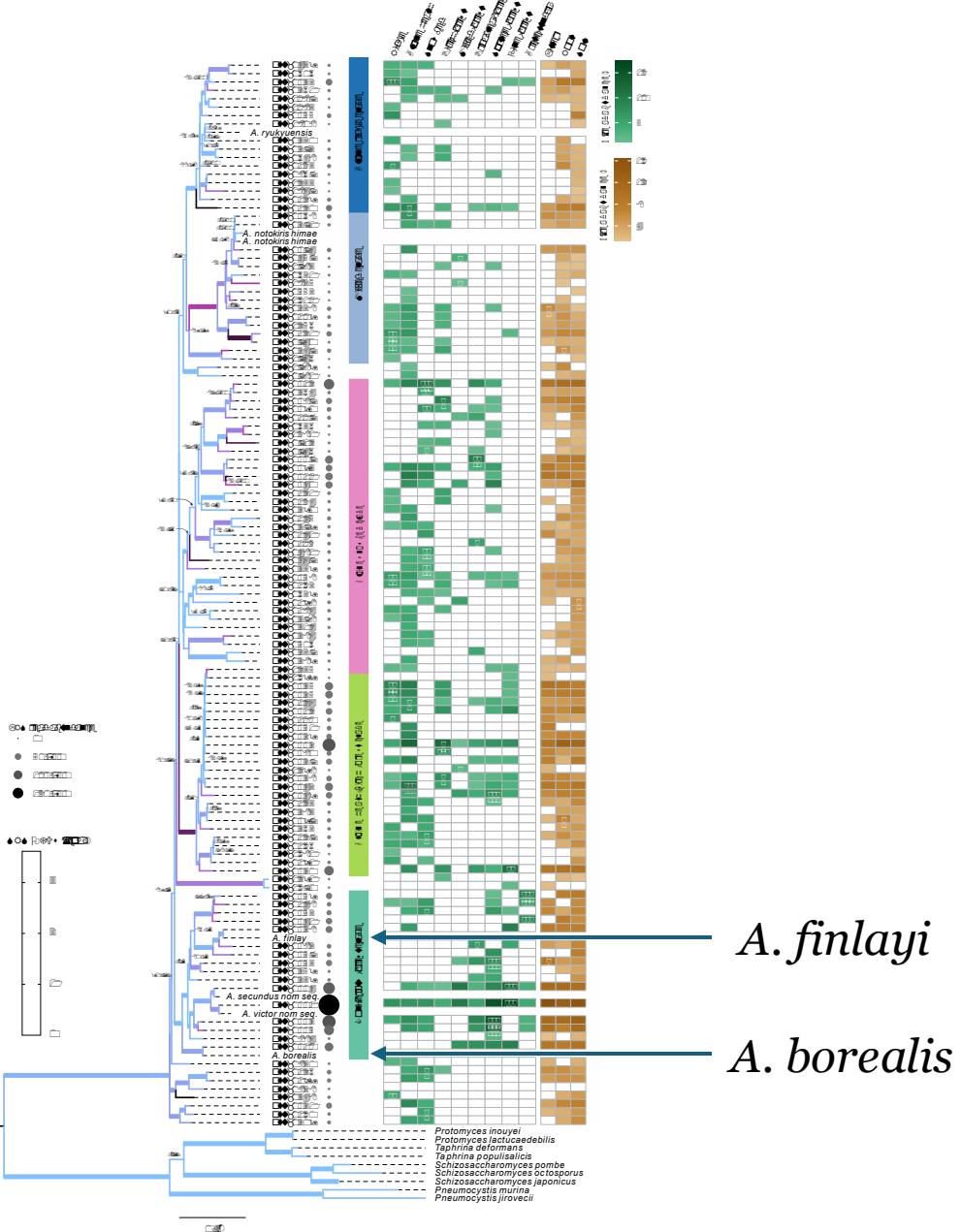


Long-read sequence data

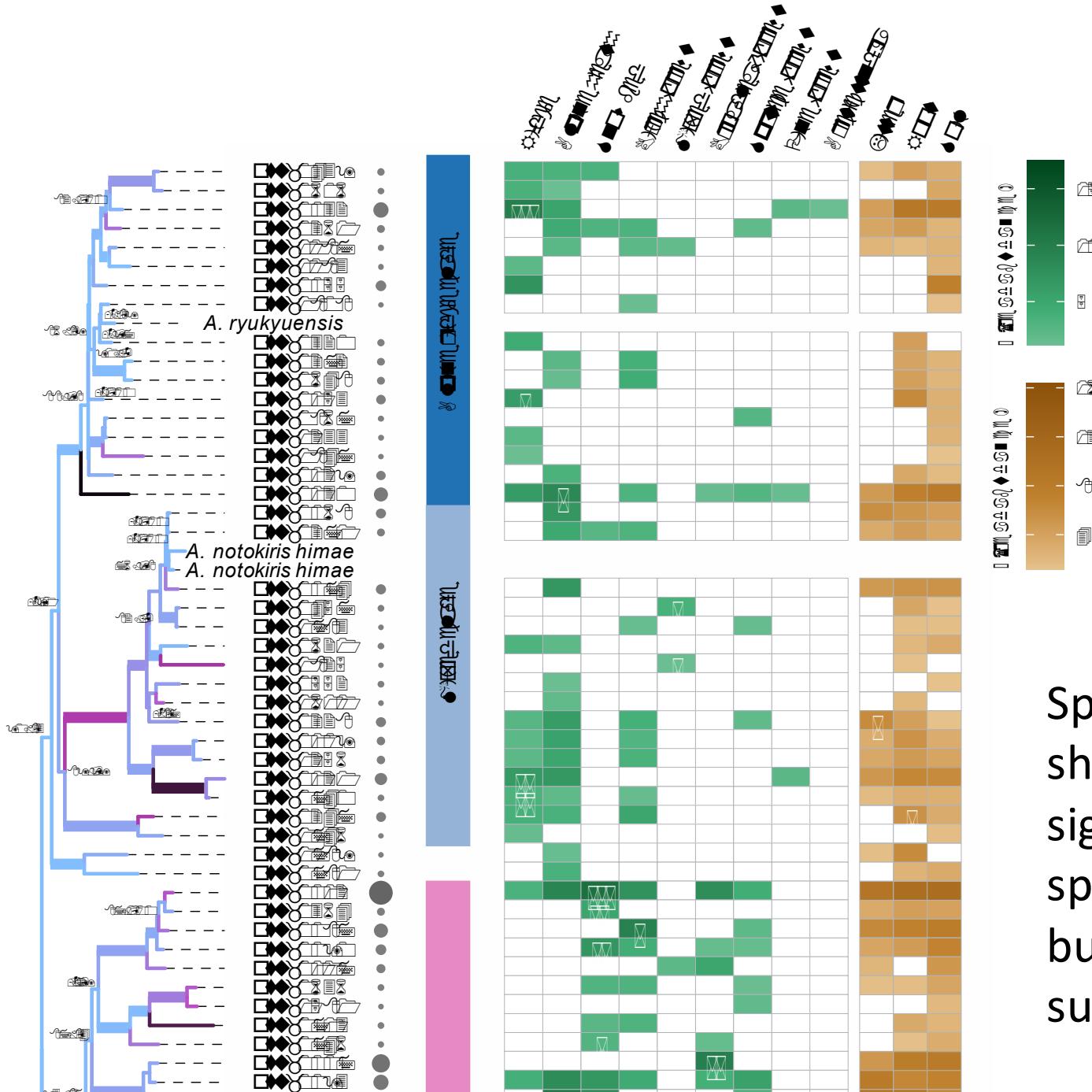


Phylogenetic distance correlated to spatial distance in the ordination
(Mantel's statistics $r=0.20$, $p=0.002$)

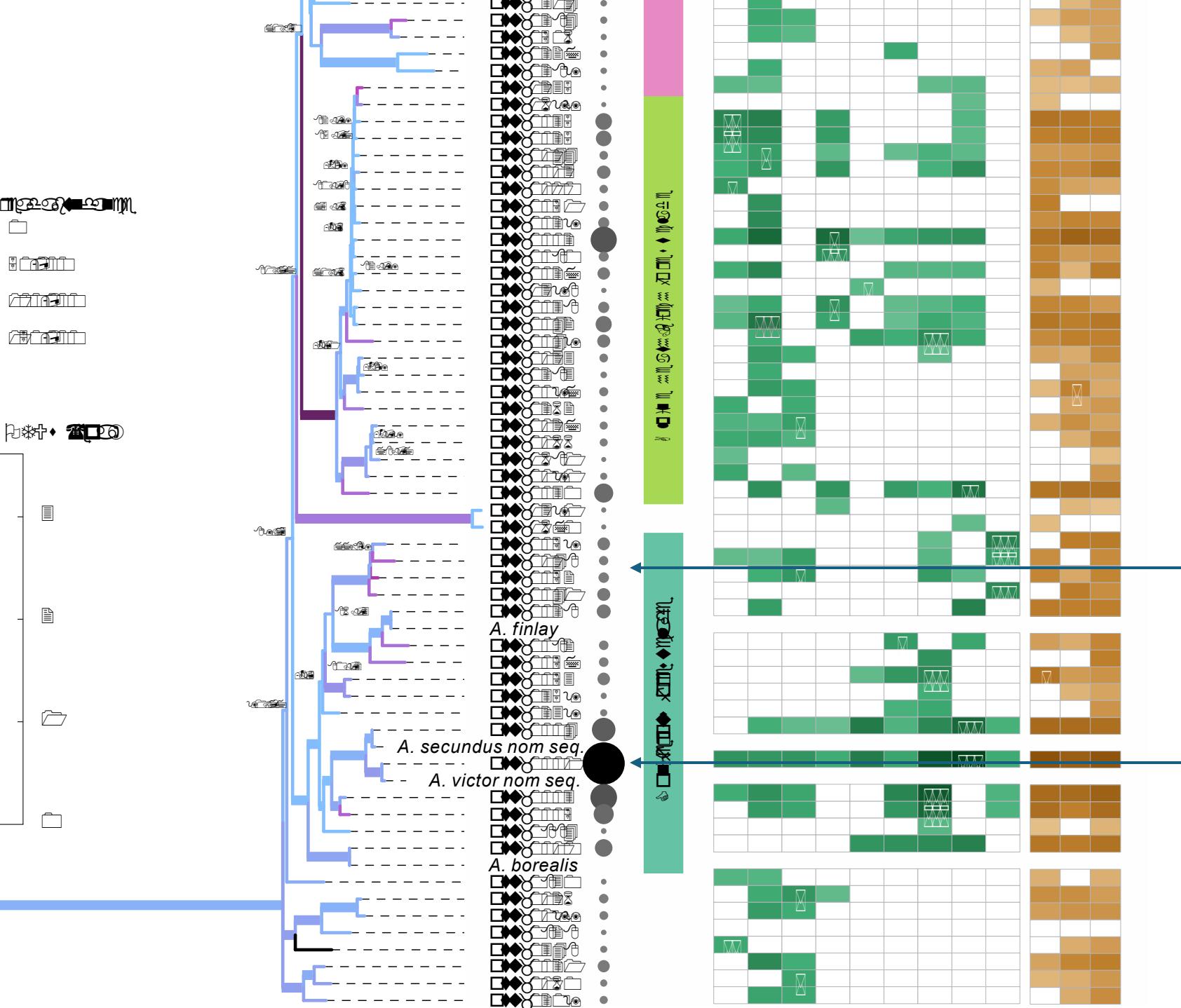
Long-read sequence data



- Clades named by the OTUs optima in the ordination space
- Relative abundances of LRS OTUs in different vegetation types (green) and substrates (brown)

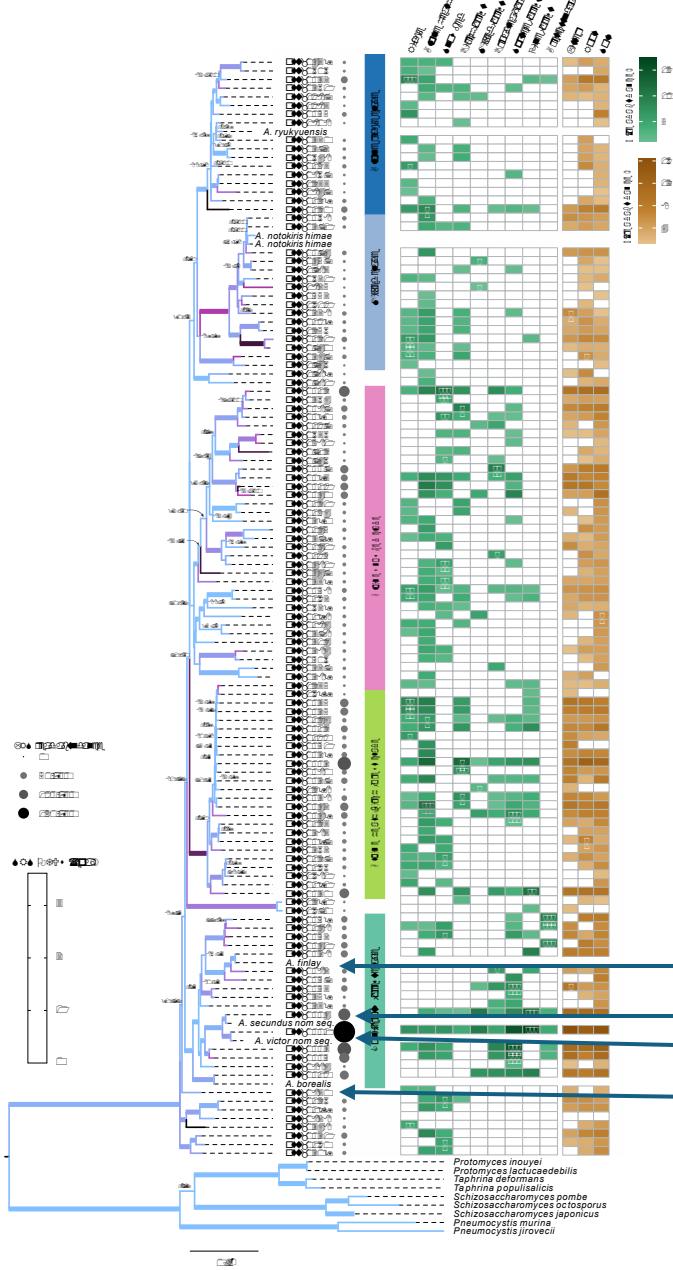


Species indicator analyses showed that 43 OTUs were significantly associated to a specific vegetation type, but only five with substrate type

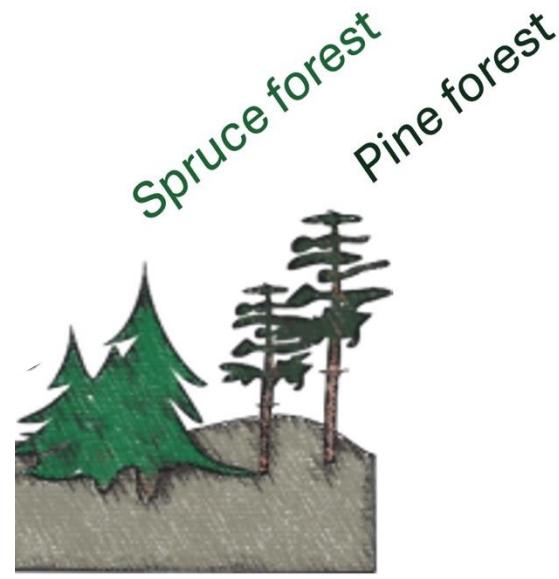


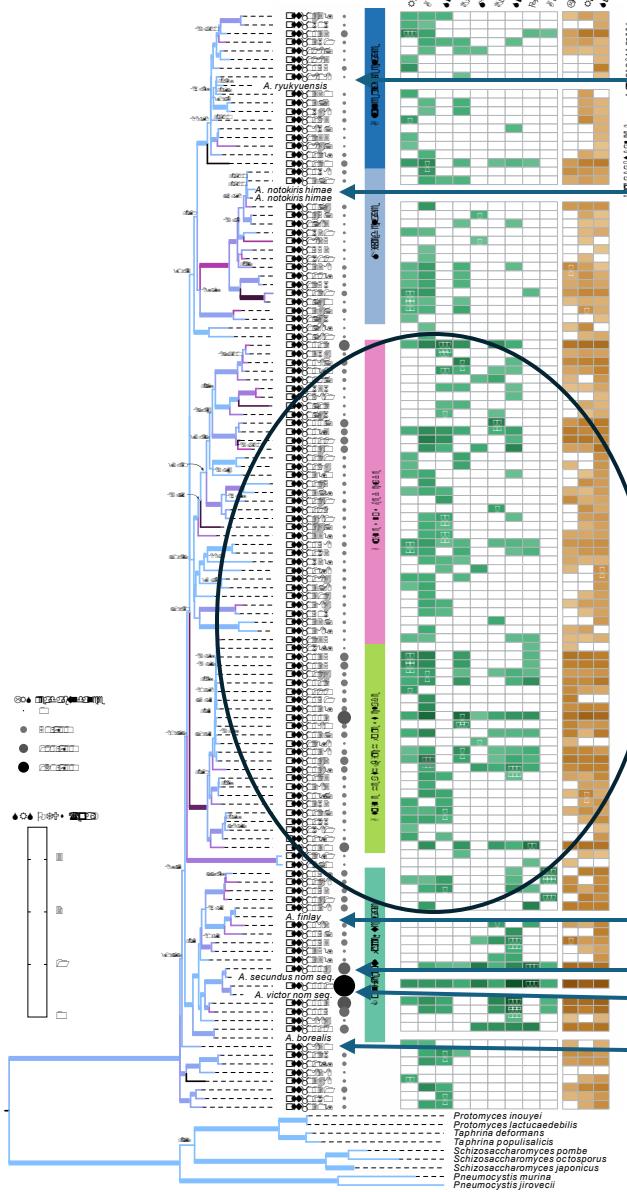
Arcic sub-clade within the boreal forest clade?

The most abundant OTU matched *A. victor nom seq.*



A. finlayi 2011
A. secundus nom. seq 2020
A. victor nom. seq 2020
A. borealis 2014





A. ryukyuensis 2024

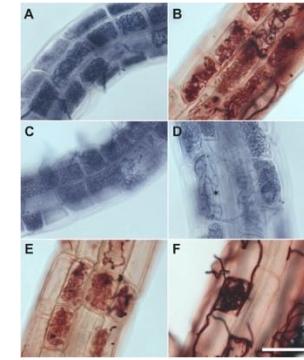
A. notokirishmae 2024

A. finlayi 2011

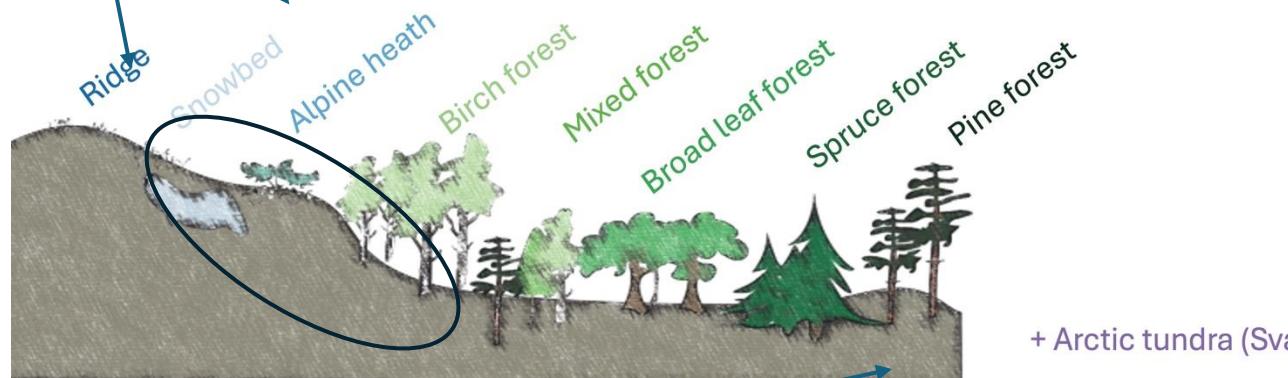
A. secundus nom. seq 2020

A. victor nom. seq 2020

A. borealis 2014



(Baba & Hirose, 2024)



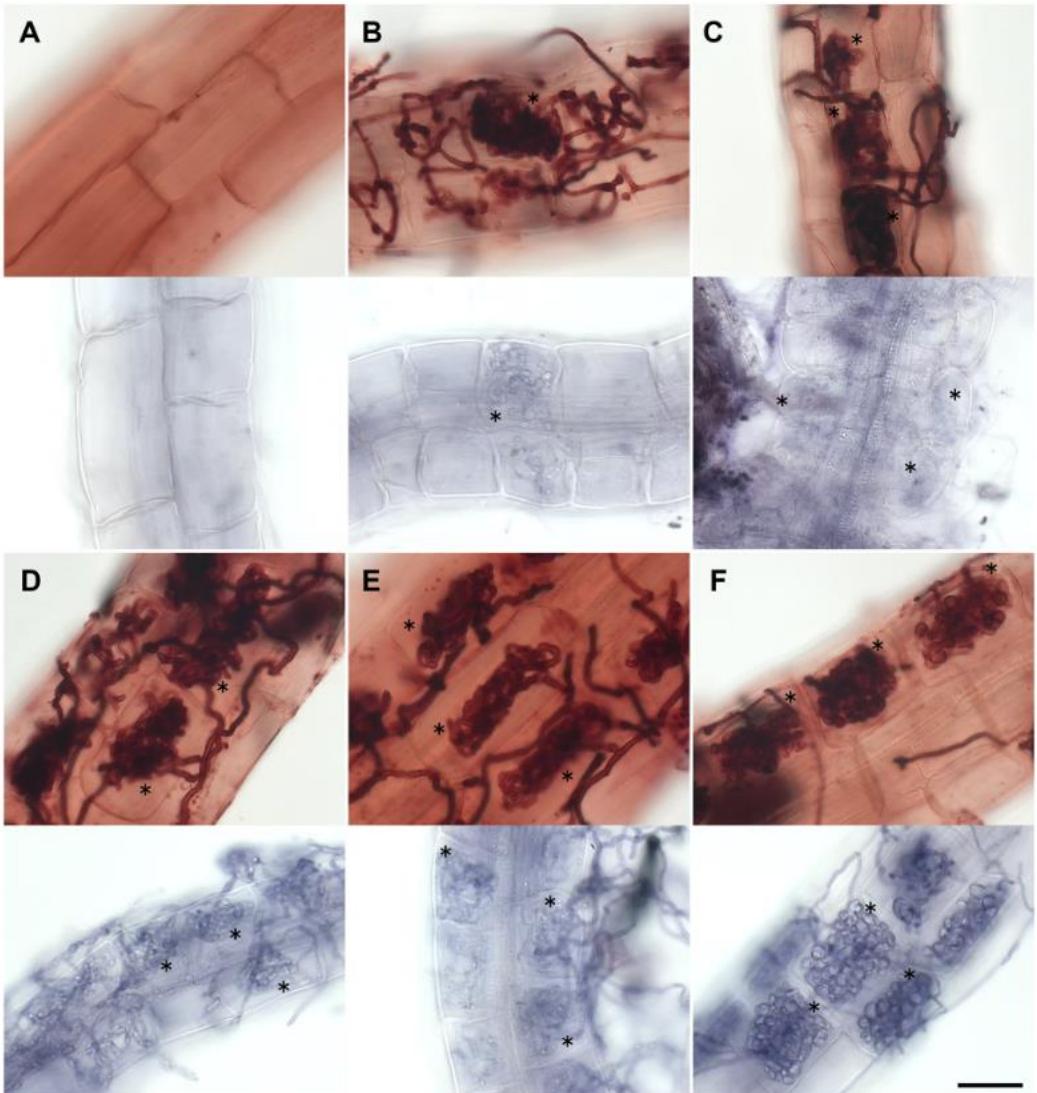
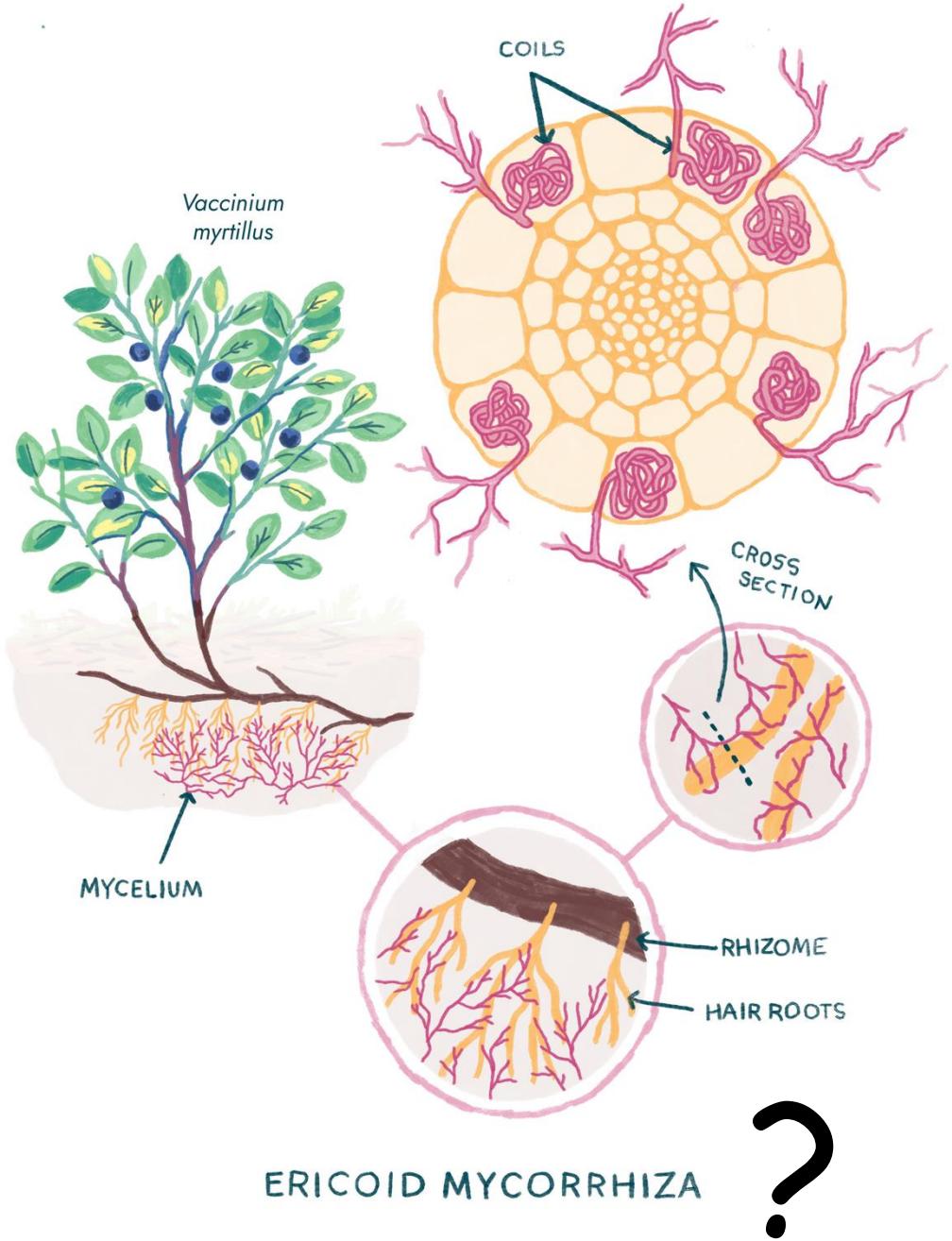


Fig. 9. Non-colonized rhizodermis (non-inoculated control, A) and rhizodermal colonization in the hair roots of *Rhododendron kaempferi* by *Hyaloscypha hepaticicola* (EF884, B), *Oidiodendron maius* (EF1409, C), *Archaeorhizomyces notokirishimae* (NBRC 116040, D, NBRC 116041, E) and *A. ryukyuensis* (NBRC 116042, F). Images of the samples stained with 3,3'-diaminobenzidine and trypan blue are shown on top and bottom, respectively. Representative rhizodermal cells containing coil is indicated with asterisks. Scale bar: 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



Short-read sequence data



Nutrient poor ridge and heath (alpine)



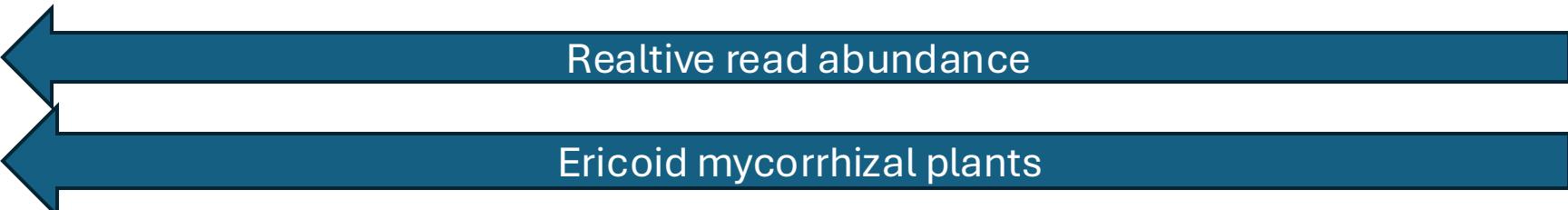
Nutrient poor forest with
ericaceous understory



Nutrient rich, moist
broad leaf forest



Beech forest (planted)



Summary & Future perspectives

- Long-read sequencing is highly suitable to explore fungal phylogenetic diversity in understudied groups using groups-specific primers
- Archaeorhizomycetes were structured by vegetation type – plant root associations?
 - But not more proportionally abundant i roots than soil
- We need more cultures of Archaeorhizomycetes!
 - We need more functional (experimental) studies to understand Archaeorhizomycetes ecology
 - But also long-read sequencing surveys mapping global diversity of the Archaeorhizomycetes
- Further work to develop and adjust long-read metabarcoding bioinformatic pipelines are needed

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