Barcoding Swiss lichens and associated fungal communities using 454 pyrosequencing

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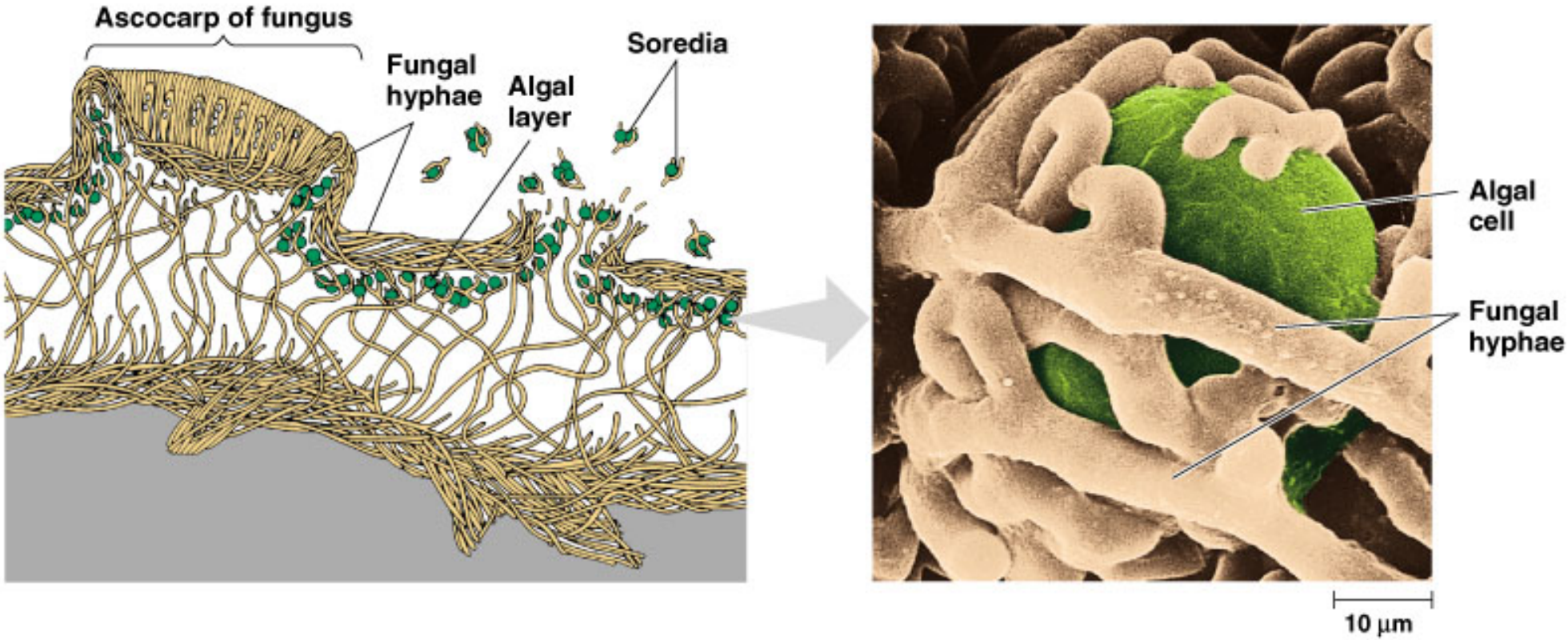
Lichens

- Symbiotic organisms consisting of a fungal partner (mycobiont) and one or more photosynthetic partner (photobiont)
- Nomenclature: Name of fungus = name of lichen



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Macrolichens: Foliose and fruticose growth forms

Hypogymnia physodes

Cladonia stellaris

Usnea dasypoga

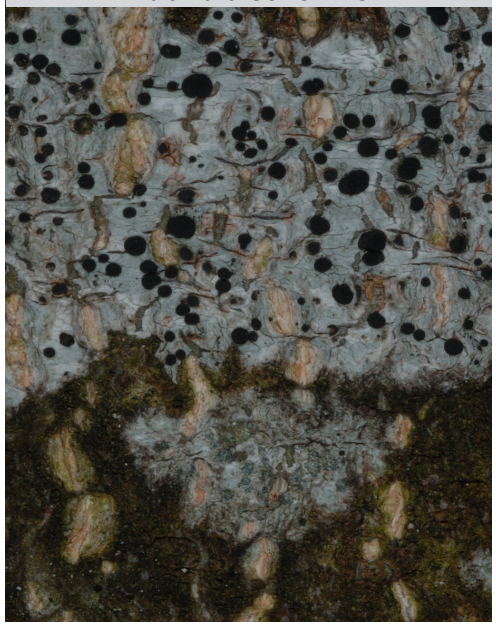
Photos: University of Tartu ©

Crustose lichens: Tightly appressed to or embedded within the substrate

Candelariella reflexa

Lecidella elaeochroma

Buellia disciformis

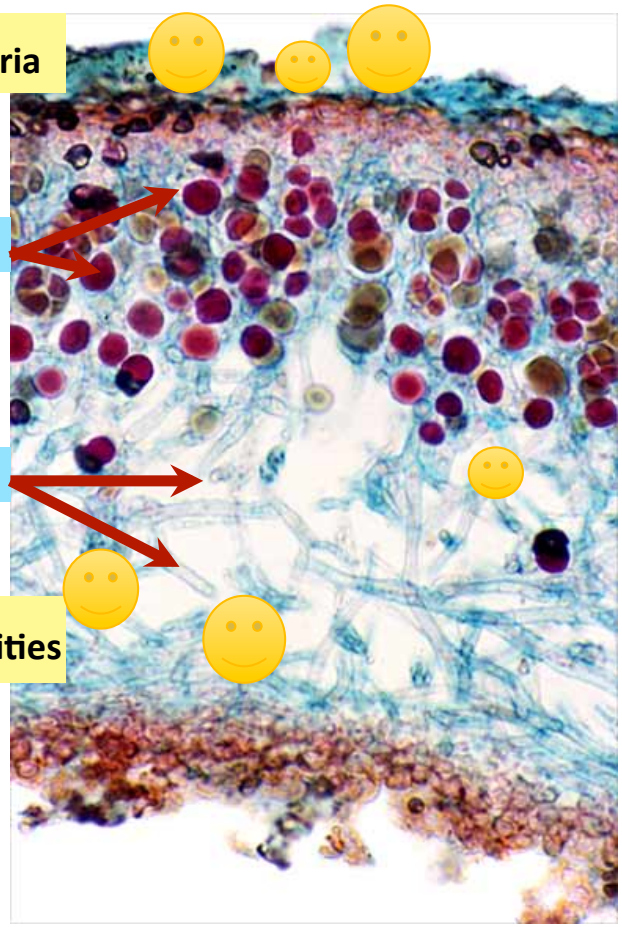


Photos: WSL ©

Why is barcoding lichens difficult?

Saprophytic, endophytic and parasitic fungi live intimately admixed with the lichen mycobiont

EPIPHYTES: free living algae, fungi, bacteria

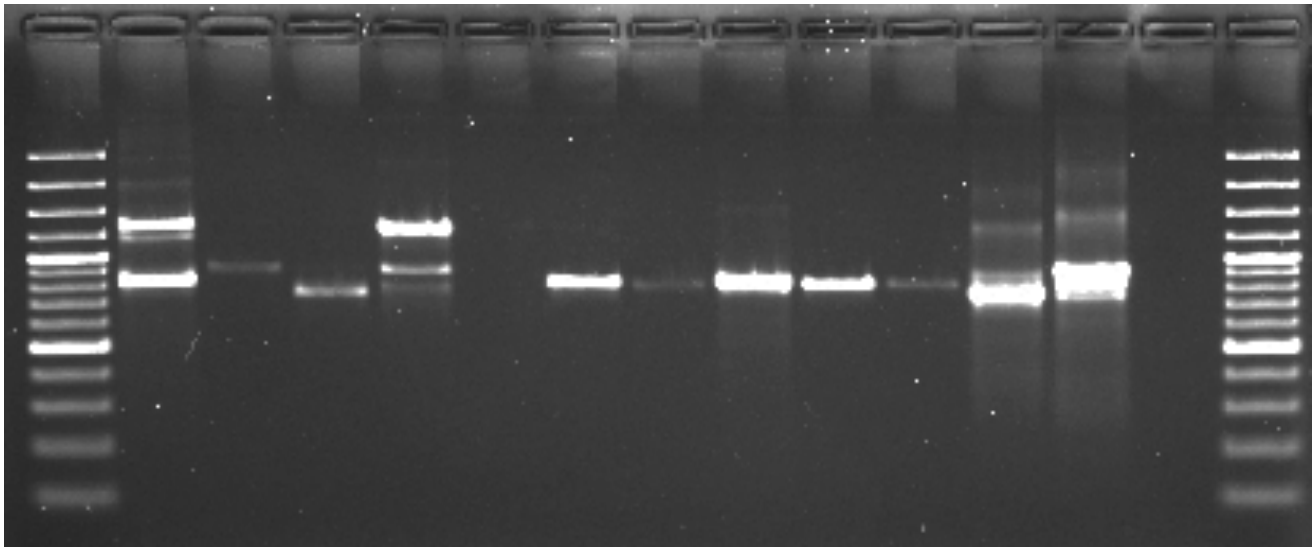


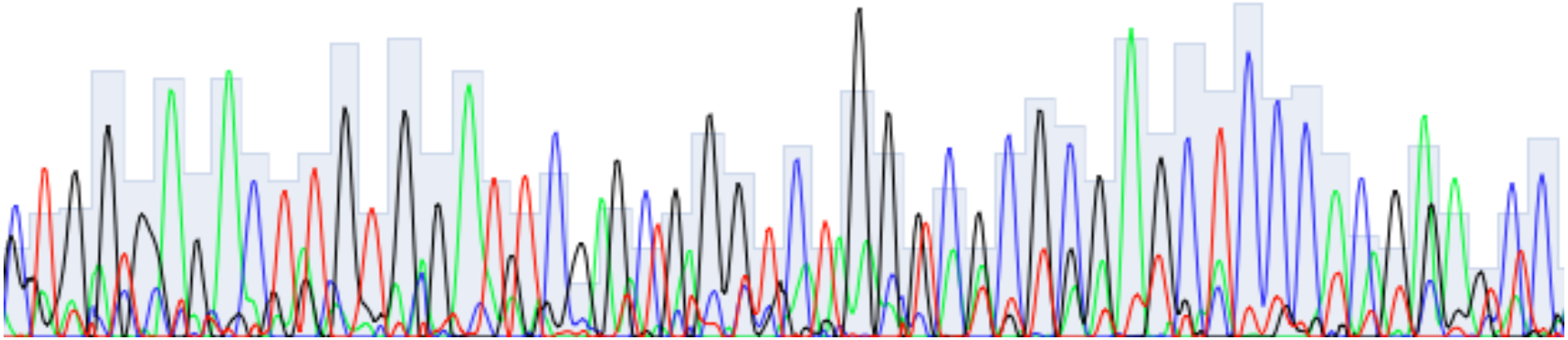
ENDOPHYTES: lichen-associated fungi, distinct bacterial communities

Cross-section photograph by Bill Malcolm

Sanger sequencing

Multiple fungi in PCR mixture result in lower quality sequences





Aim of the study:

Testing barcoding of the lichen mycobiont and associated fungi

- 100 lichen species;
- collected from Switzerland;
- using fungal specific molecular markers;
- in high-throughput 454 sequencing in GS FLX System.

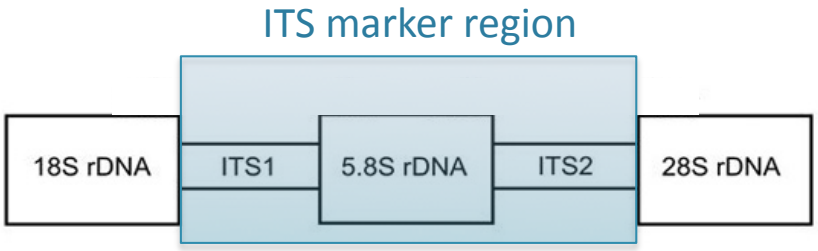
Molecular methods

[1] DNA extraction

[2] PCR amplification

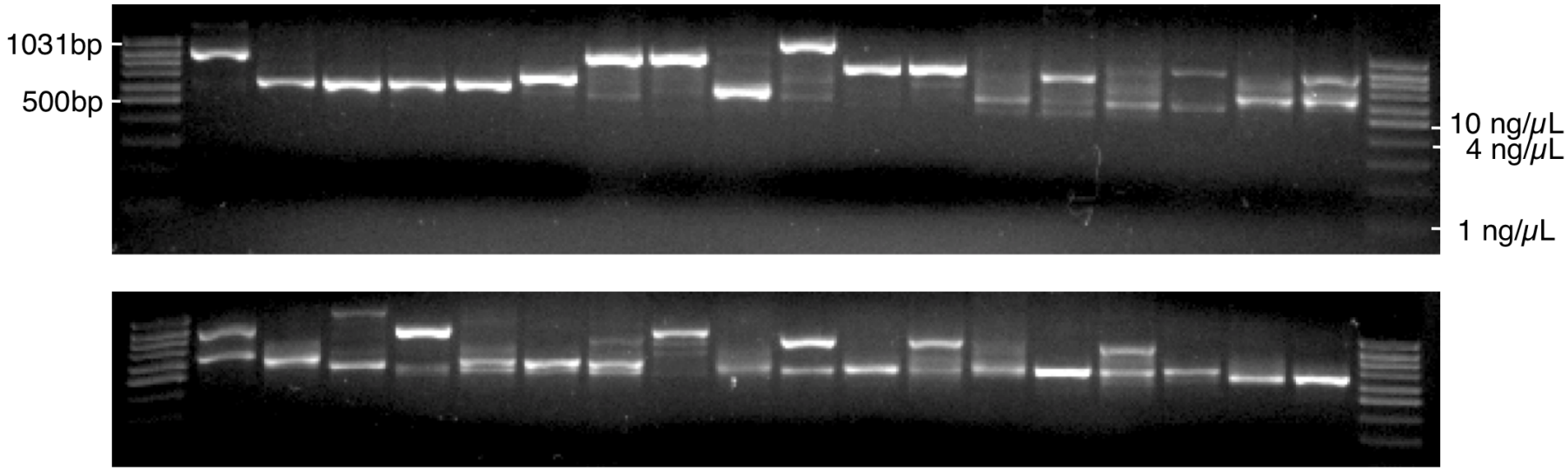
Hi-Fidelity polymerase

Standard fungal specific ITS primers: ITS1F & ITS4



[3] Amplicon sequencing in Roche/454 GS FLX+ system

GEL EXAMPLE



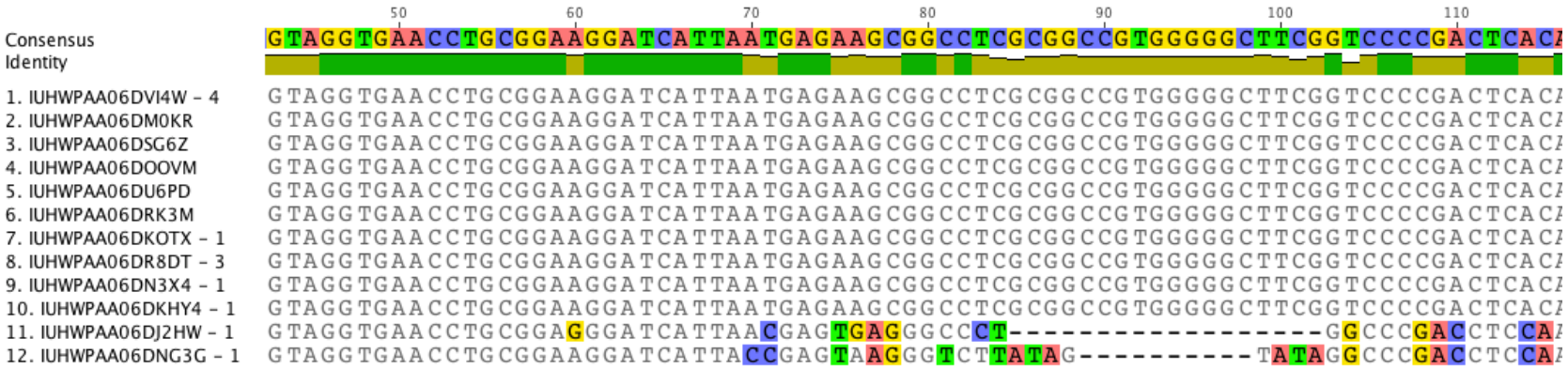
454 Data Analyses

454 Data Analyses

INITIAL RESULTS

↓

Generating target taxon consensus sequence = “barcode”



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FINAL RESULTS: Confirming the “barcode” in NCBI megablast search

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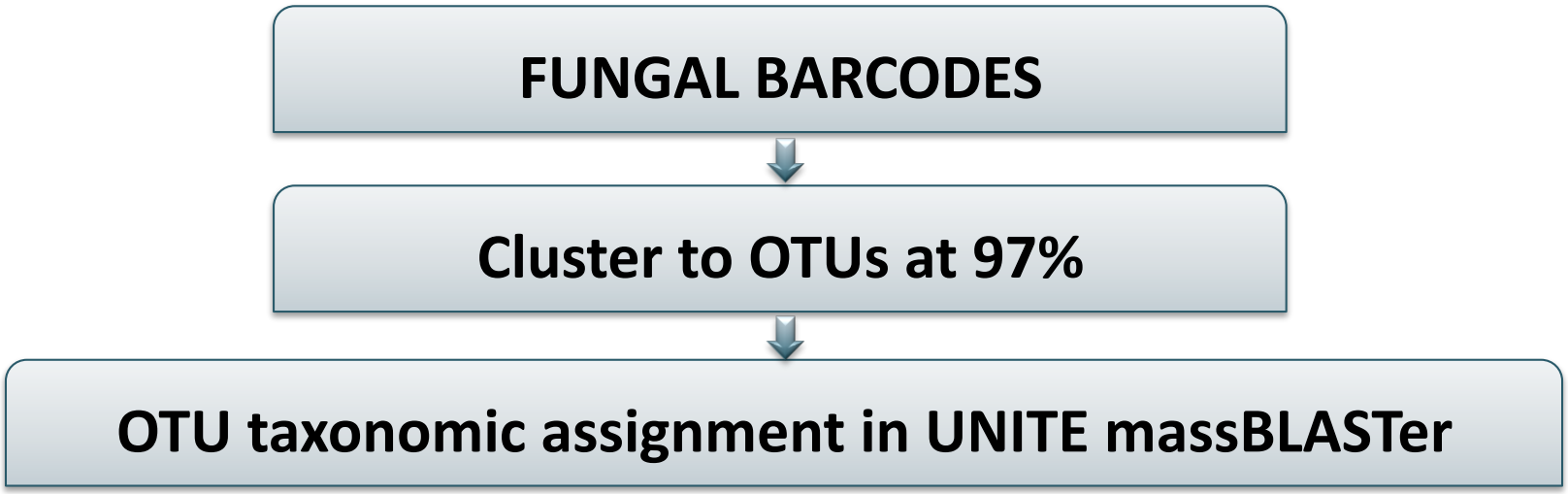
Alignment and tree-based identification

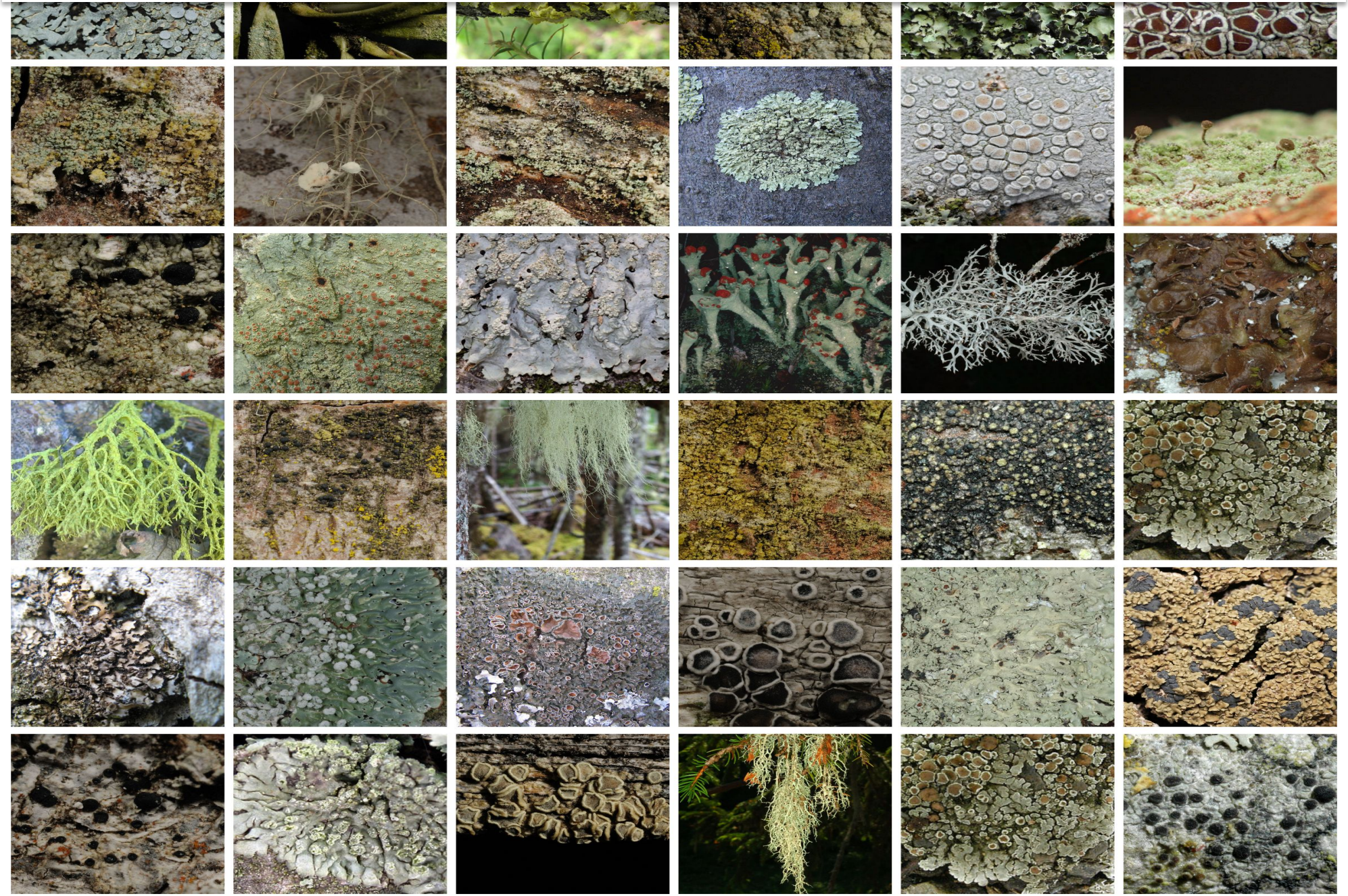
454 Data Analyses



Results: PCR & Sequencing

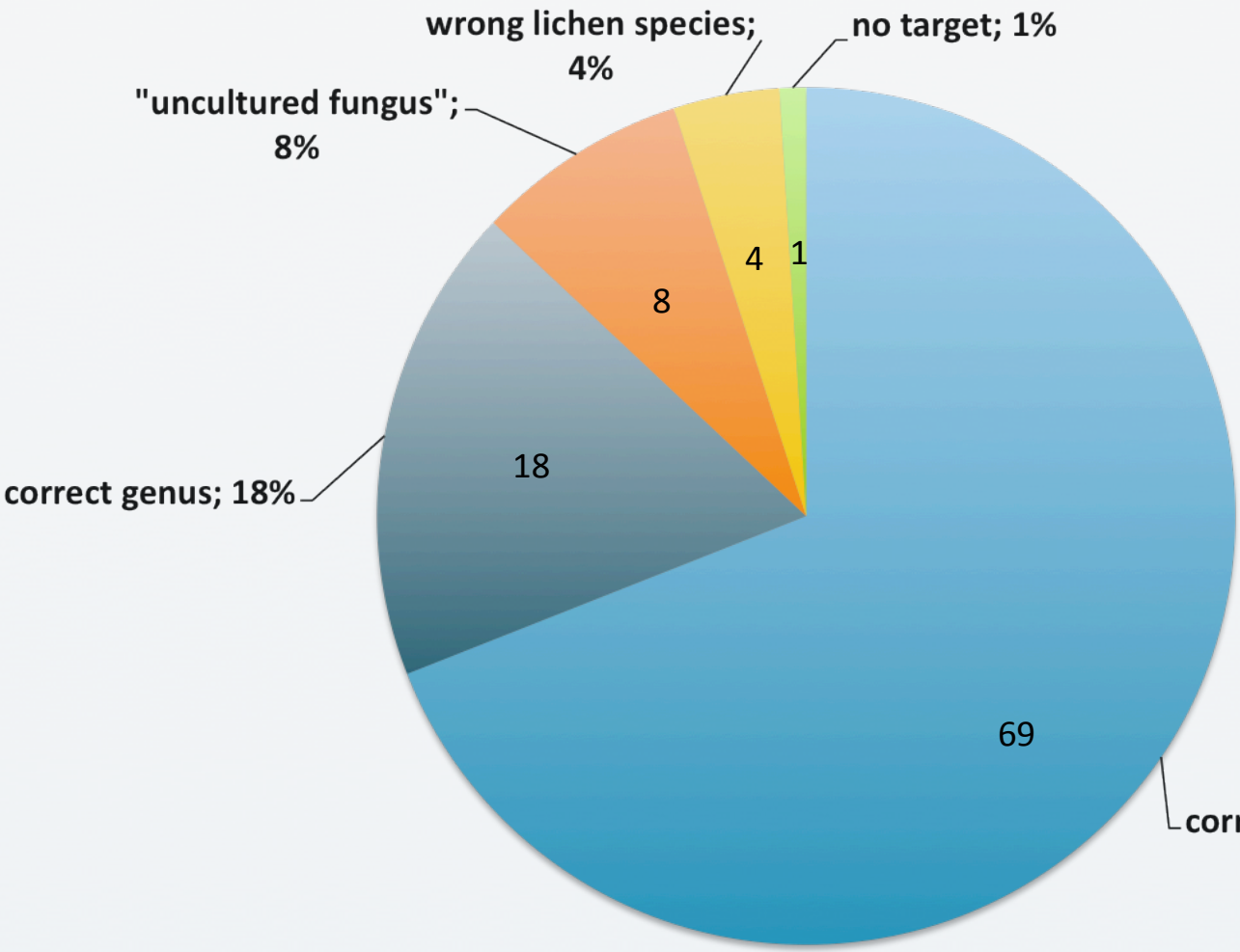
PCR products	100
Sequences total	128 449
Mean no of reads per sample	1285
Average length (bp)	615
Average quality score	32.8
Average no of clusters per sample at 95%	88
Barcodes	1171
Fungal OTUs	567

Barcoded lichen diversity



Results: Lichens

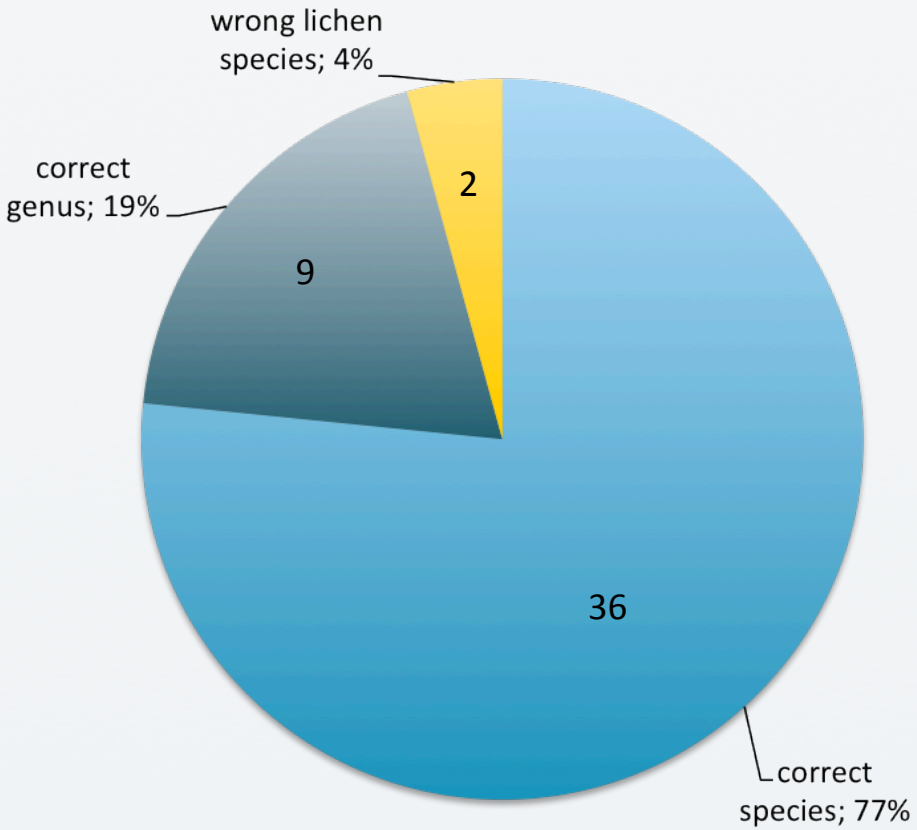
Target taxon identified to …



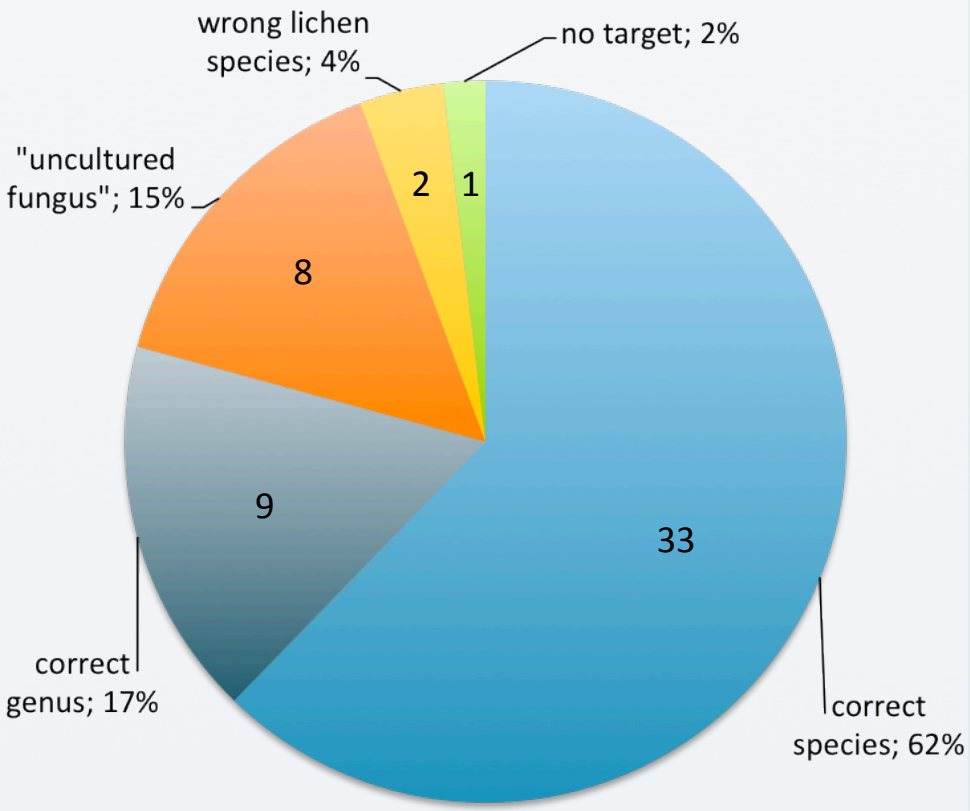
Results: Lichens

Target taxon identified to …

FOLIOSE/FRUTICOSE LICHENS



CRUSTOSE LICHENS



Results: Lichens

- 69 samples identified **correctly to species level**, of which 60 with **similarity >97%**
- 9 samples identified to **correct genus but wrong species** with **similarity >97%**
  - → EXAMPLE 1: *Usnea*
  - → EXAMPLE 2: *Anaptychia*
- 1 sample identified to **wrong lichen species** with **similarity >97%**
  - → EXAMPLE 1: *Parmelia sulcata*

Examples: *Usnea*

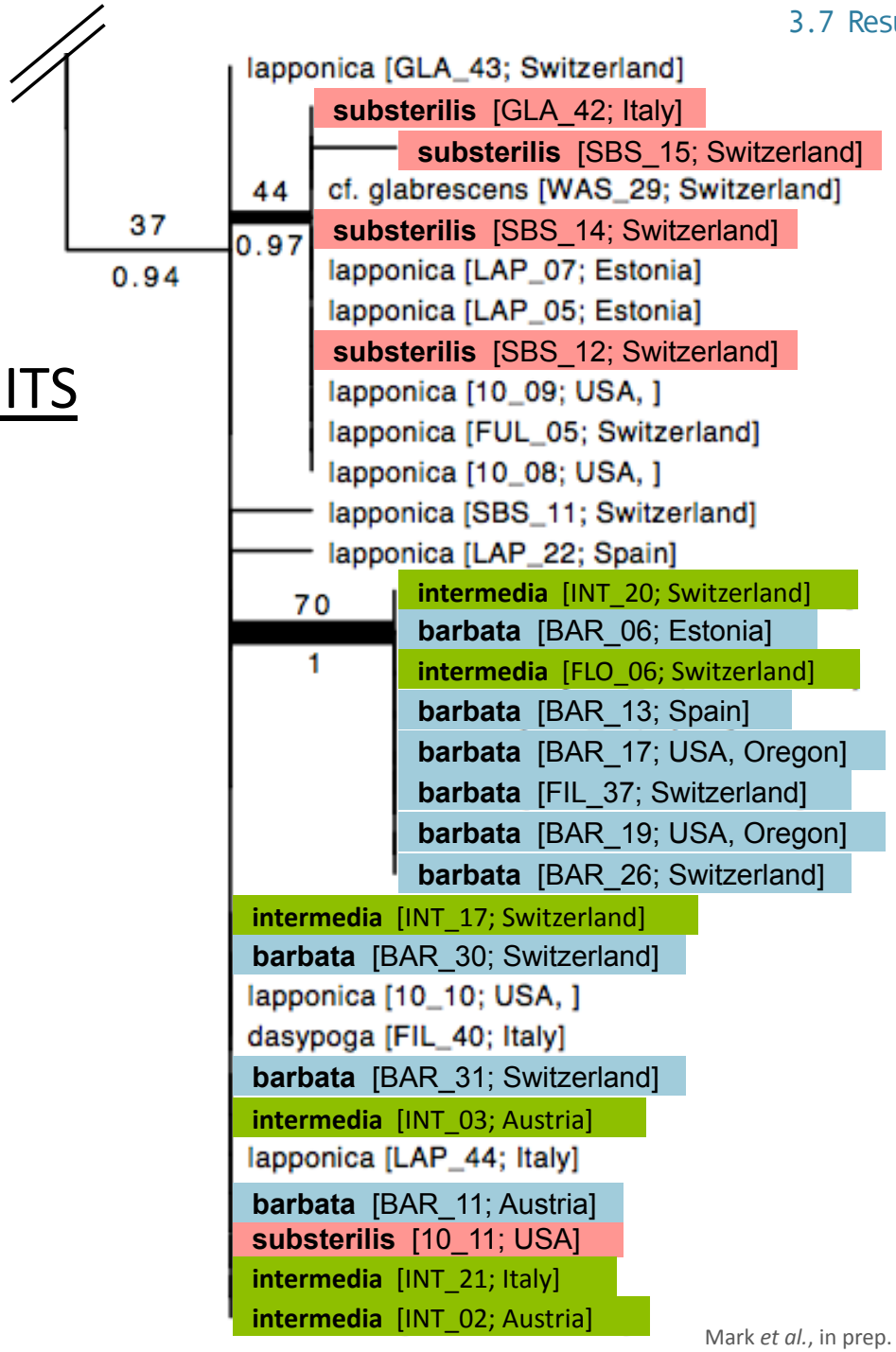
NCBI BLASTN

1) KM-03-01 Usnea intermedia ➔ AJ457152 Usnea rigida 99.8%
2) LC-011 Usnea barbata ➔ AJ457152 Usnea rigida 100%
3) LC-009 Usnea substerilis ➔ AJ457152 Usnea rigida 98.7%

Photos: University of Tartu ©

Examples: *Usnea*

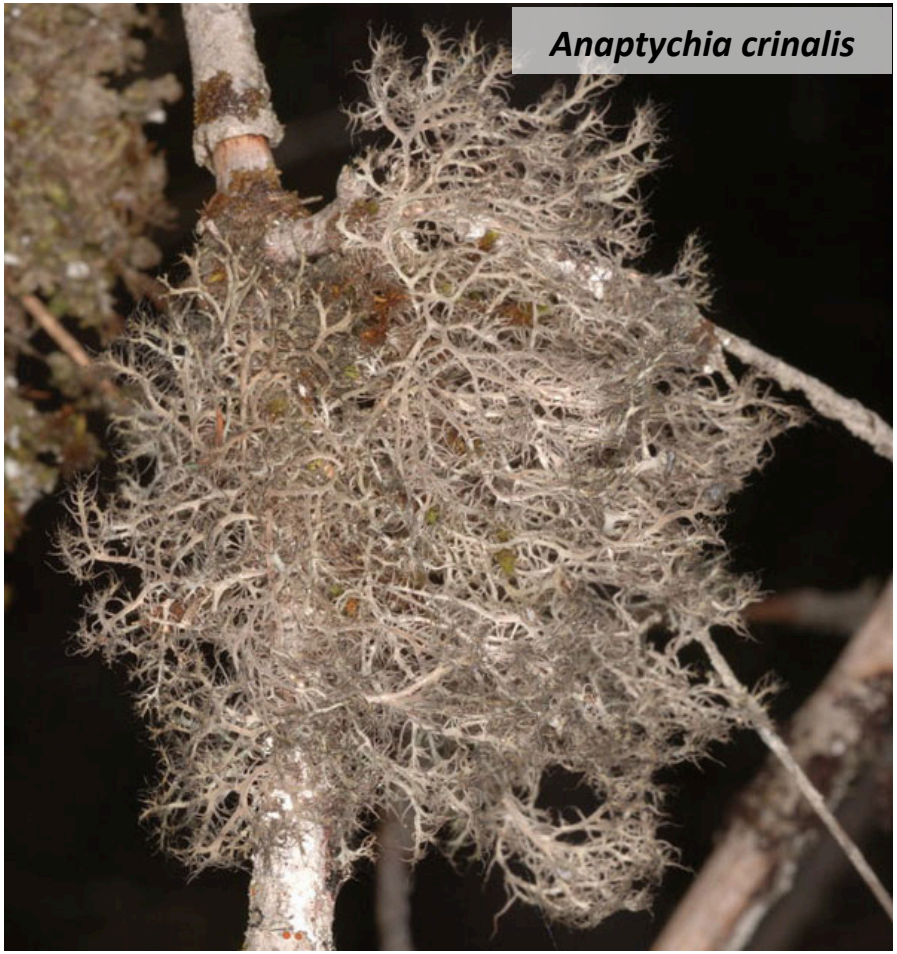
- LOW GENETIC VARIATION in ITS

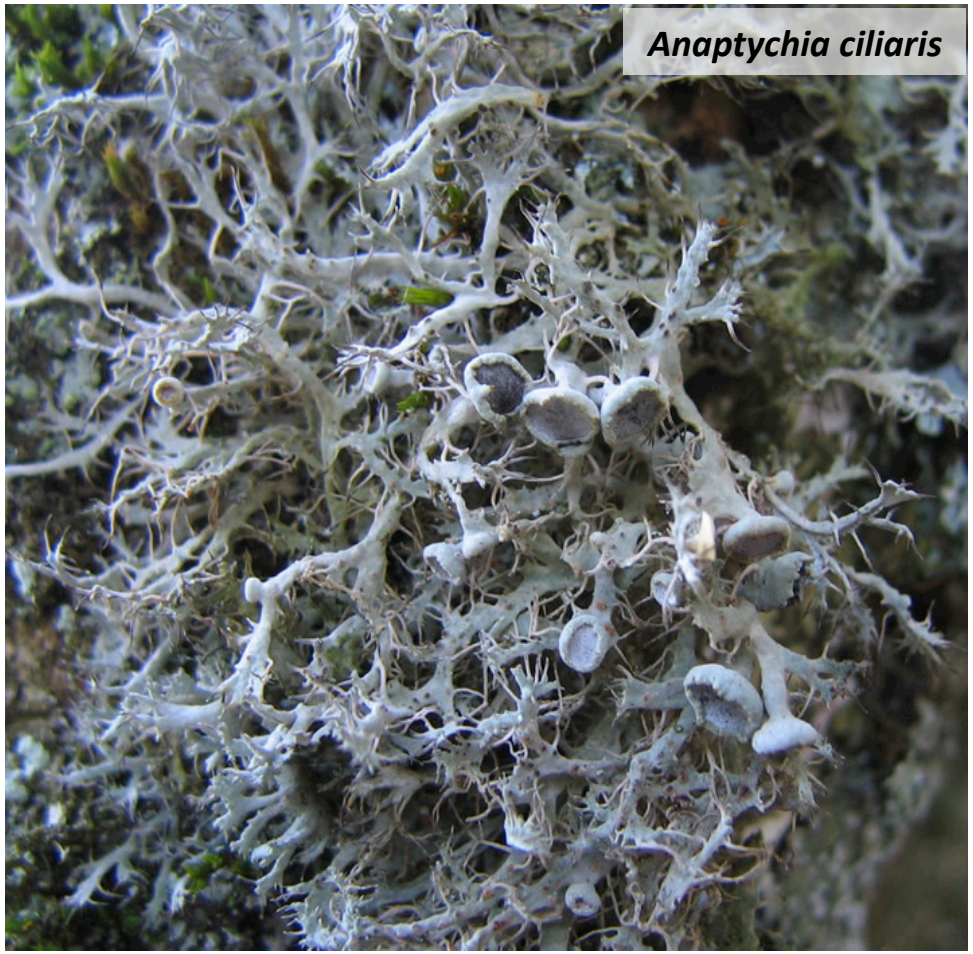


Mark *et al.*, in prep.

Examples: *Anaptychia crinalis*

LC 071 Anaptychia crinalis ➔ KJ027716 Anaptychia ciliaris 98%



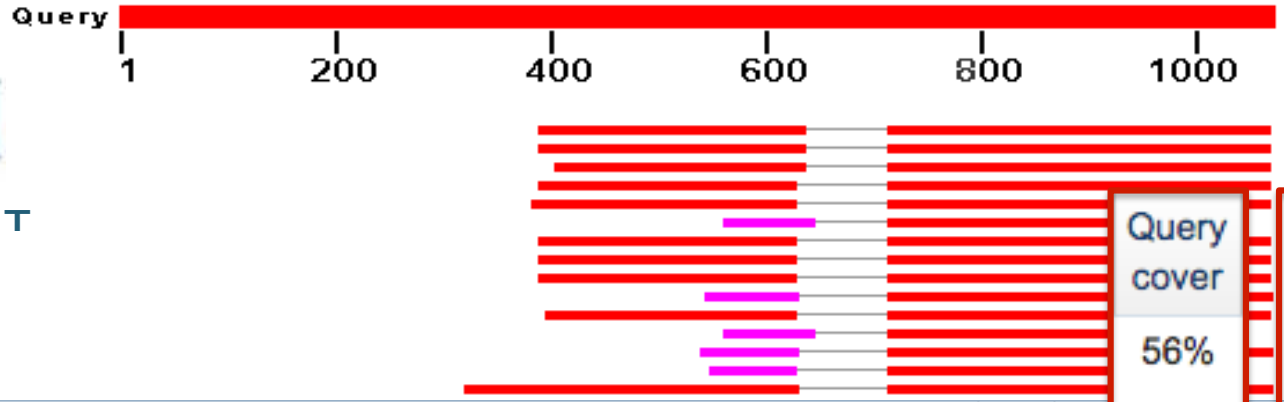


Photos: WSL ©

Examples: *Anaptychia crinalis*

❖REFERENCES DO NOT COVER THE BARCODED REGION IN SIMILAR AMOUNTS OR AREA

NCBI MEGABLAST



Description	Max score	Tota scor	E alu	Query cover	Ident	Accession
Anaptychia ciliaris isolate E3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	566	986	-15	56%	96%	J027714.1
Anaptychia ciliaris isolate E2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	566	997	-15	56%	96%	J027713.1
Anaptychia ciliaris isolate E5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	566	962	-15	54%	96%	J027712.1
Anaptychia ciliaris isolate E1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	560	901	-15	55%	95%	J027717.1
Anaptychia ciliaris isolate N5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	560	962	-15	55%	95%	J027715.1
Anaptychia crinalis isolate 67A internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcrib	555	713	-15	37%	98%	F582786.1
Anaptychia ciliaris isolate N1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	549	896	-15	55%	95%	J027718.1
Anaptychia ciliaris isolate N4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	544	868	-15	55%	94%	J027720.1
Anaptychia ciliaris isolate N2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal trans	544	890	-15	55%	94%	J027719.1

Examples: *Parmelia sulcata*

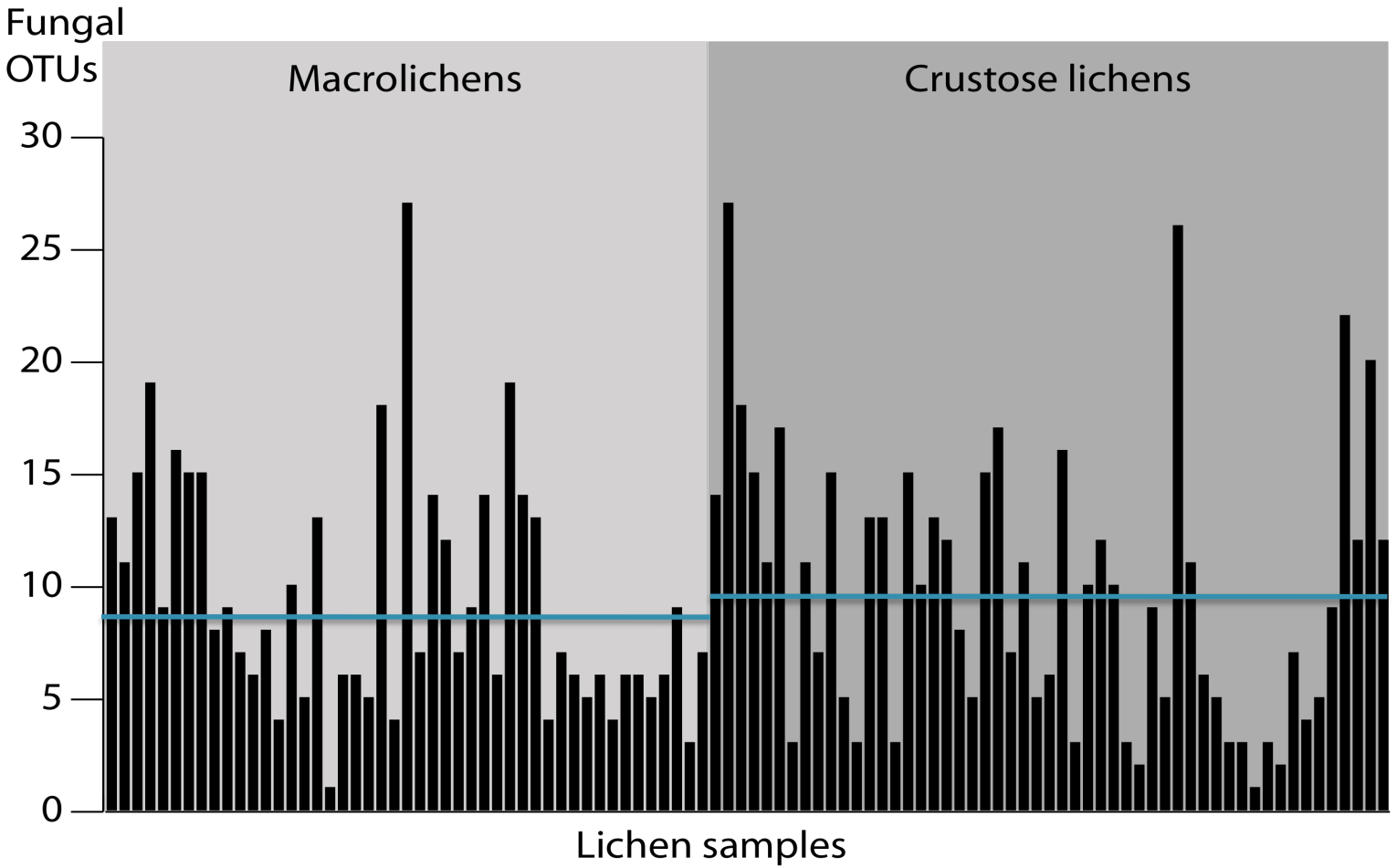
❖PROBABLE MISTAKE IN REFERENCE DATABASE

1) LC-004 Parmelia sulcata ➔ HQ671303 Myelochroa aurulenta 99.6%

- tropical species
- sequence more similar to *Parmelia sulcata* sequences (99%) than to other *Myelochroa*

High fungal diversity in lichens

On average about **10 OTUs per lichen sample**



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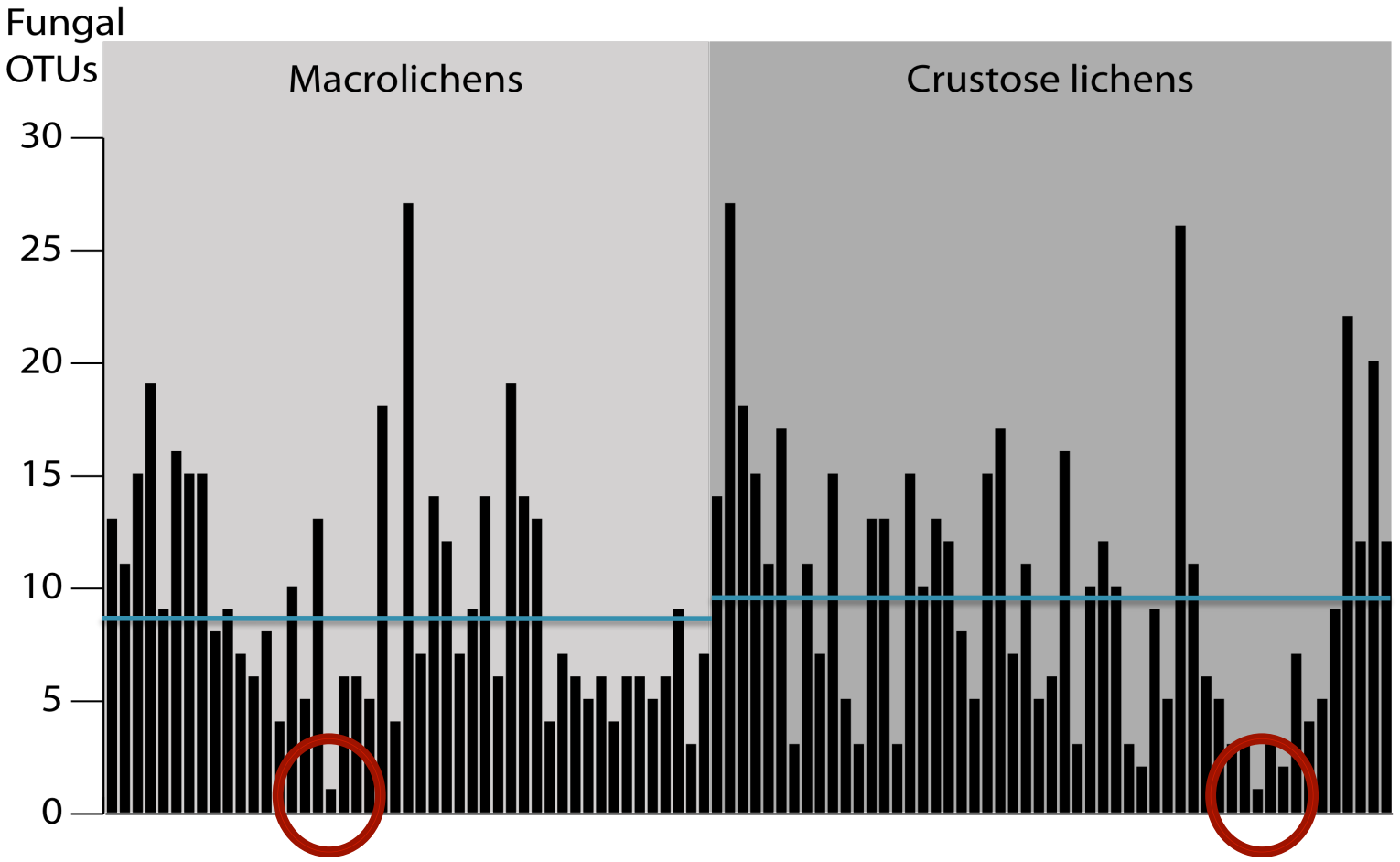
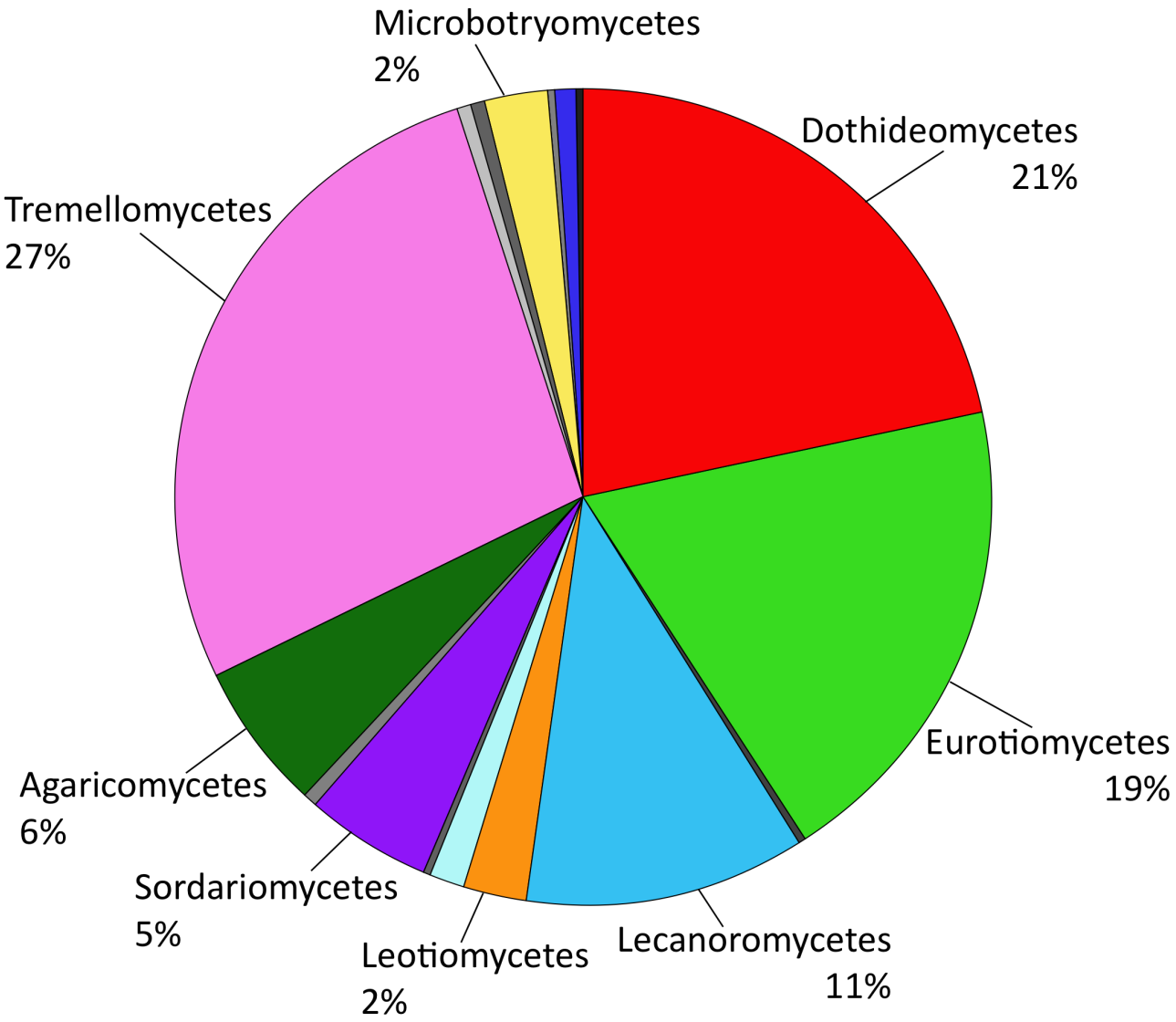


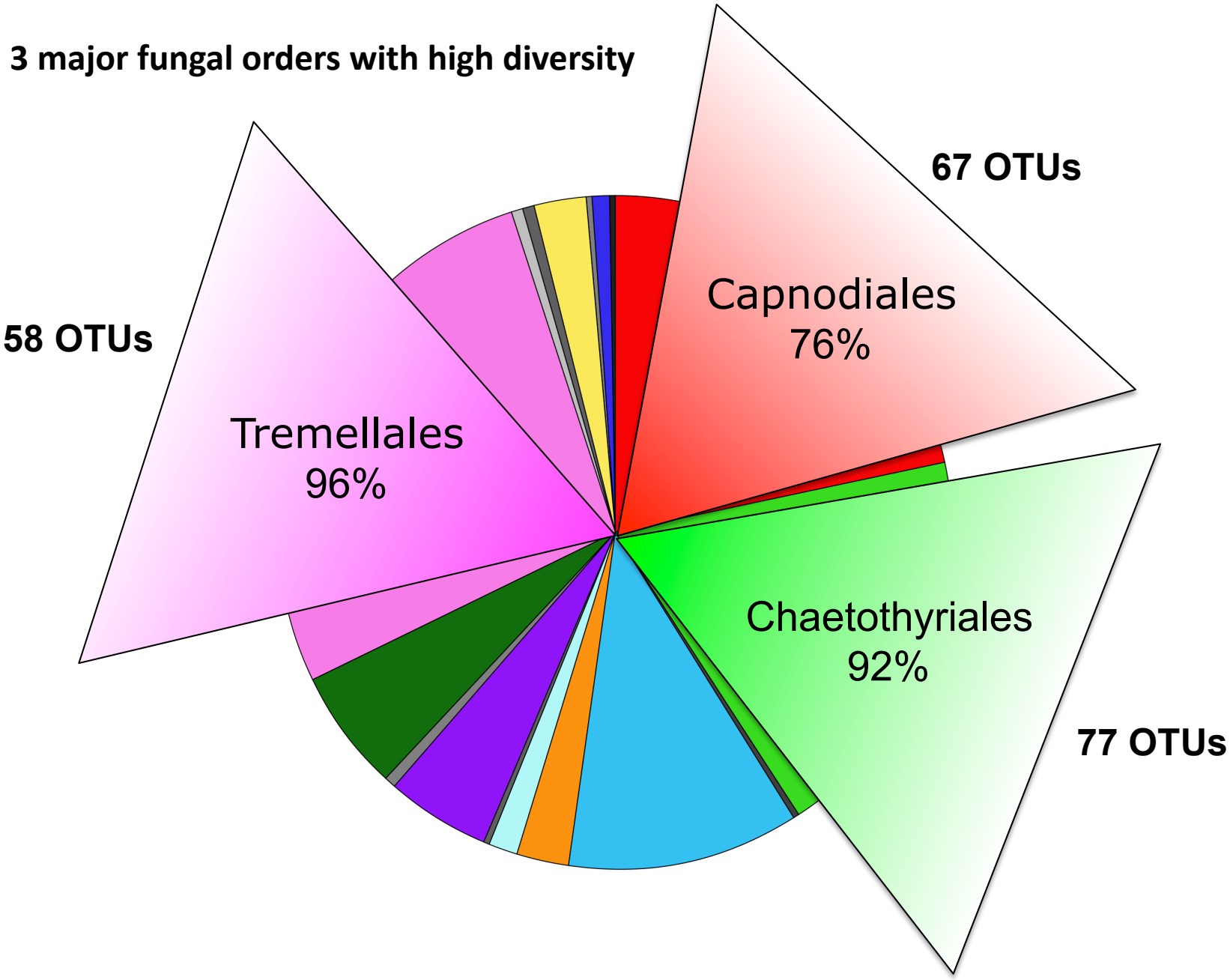
2 samples with the lichen mycobiont only

Lichen-inhabiting fungal diversity

22 different fungal classes



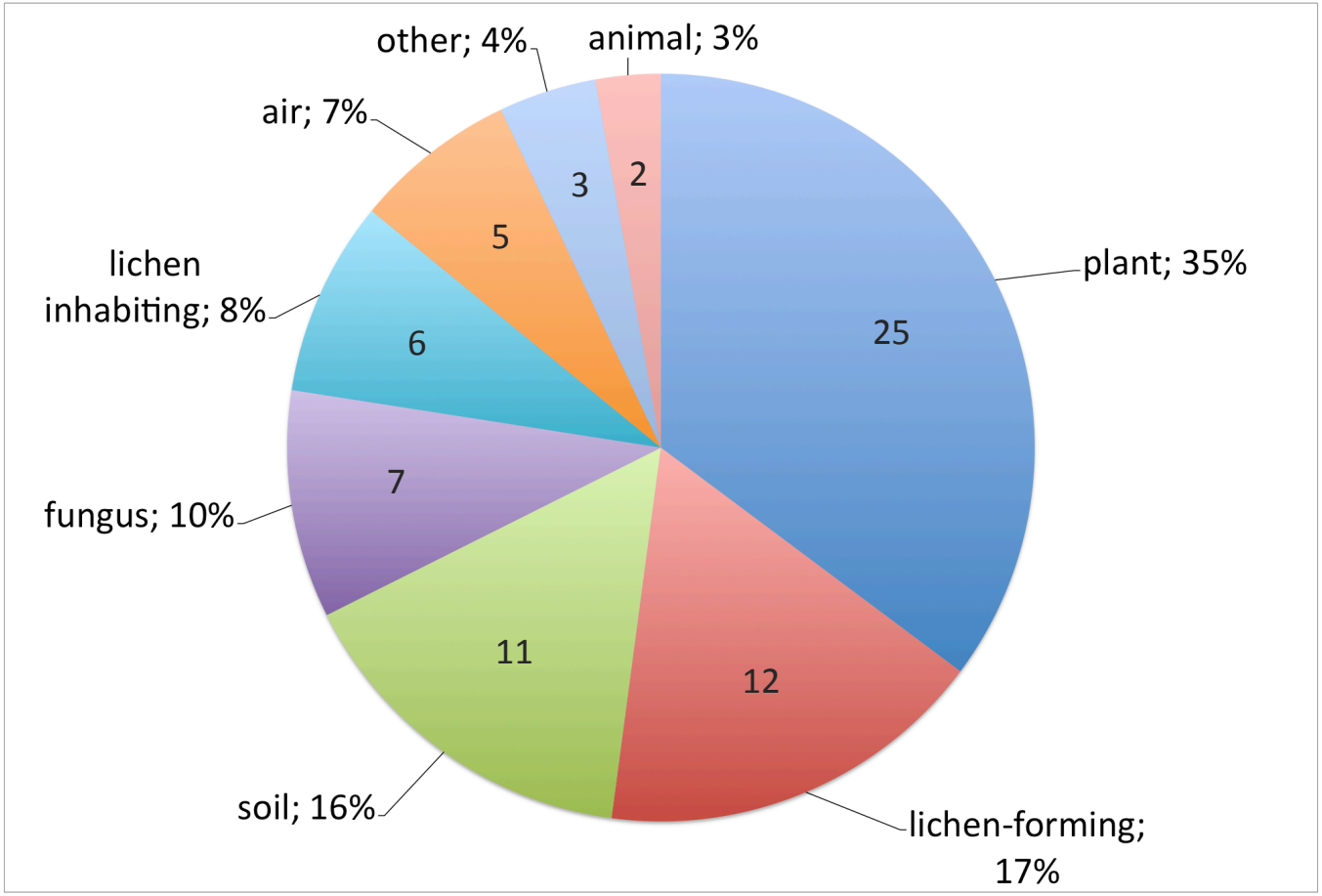
Lichen-inhabiting fungal diversity



Results: Lichen-inhabiting fungi

- **71 OTUs identified with ≥97% similarity to species level**

GenBank match isolation source:



Conclusions: Lichens

- Fungal ITS sequence ("barcode") of target lichen taxon recovered for 99 samples from 100

- In GenBank BLAST
  - 69 samples identified to species level, of which 60 with similarity >97%
  - 18 species identified to correct genus level

- Misidentifications due to
  - incomplete reference database
  - open taxonomic questions in some groups
  - labelling mistakes

Conclusions: Lichen-inhabiting fungi

- For 22 samples the most sequenced fungus was not the expected lichen mycobiont

- Many other fungi recovered within samples — on average 10 fungal lineages per lichen sample

  - lichen-associated (lichenicolous/facultative parasites)
  - plant-associated (epi- or endophytes)
  - generalists (previously isolated from various sources, e.g. soil, air, fungi, plants, animals)

Thank you!

6th International Barcode of Life Conference
Barcodes to Biomes

- Financial support:

  Sciex-Project 13.059

  SwissBOL

- **Personal thanks:**

  Jean-Claude Walser, Tiina Randlane, Andres Saag, Ave Suija, Polina Degtjarenko

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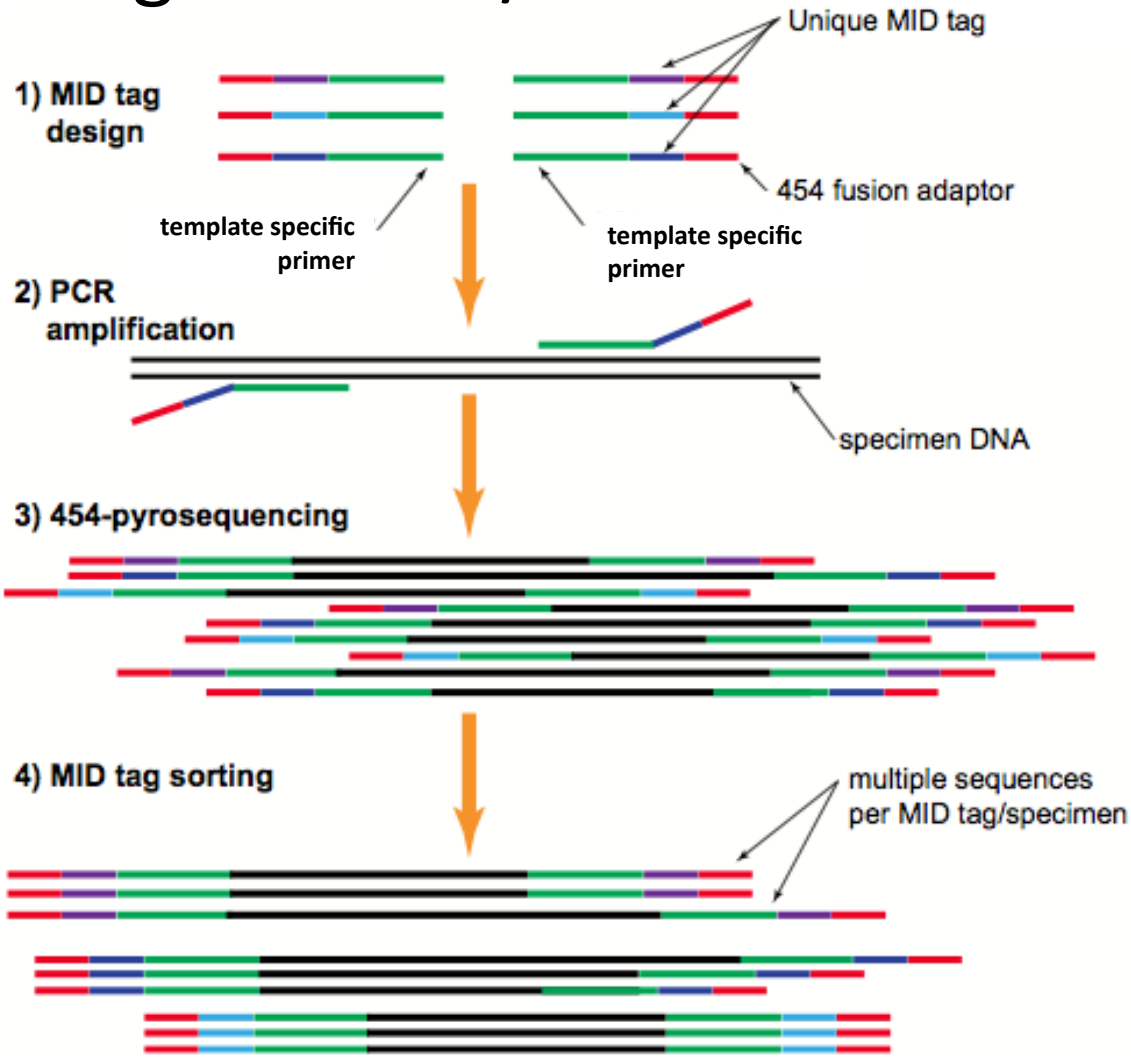
Rektorenkonferenz der Schweizer Universitäten
Conférence des Recteurs des Universités Suisses
Conferenza dei Rettori delle Università Svizzere
Rectors' Conference of the Swiss Universities

Molecular methods

[3] Amplicon sequencing in Roche/454 GS FLX+ system

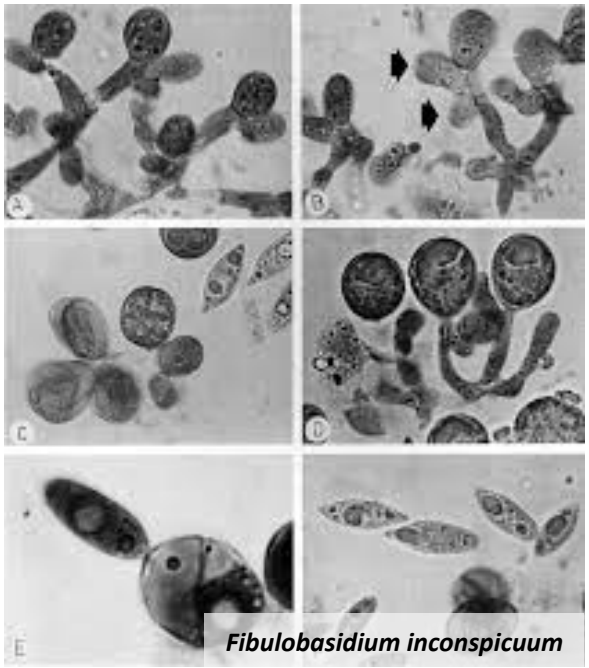
1. 25 unique MID tags – "barcodes"
2. 2nd step PCR with 25 barcoded fusion primers
3. Purification, quantification, and equimolar pooling in four pools of 25 PCR products each
4. Sequencing on 4/16 run of the FLX instrument with the FLX+ reagents
5. MID tag sorting



Shokralla et al. 2014

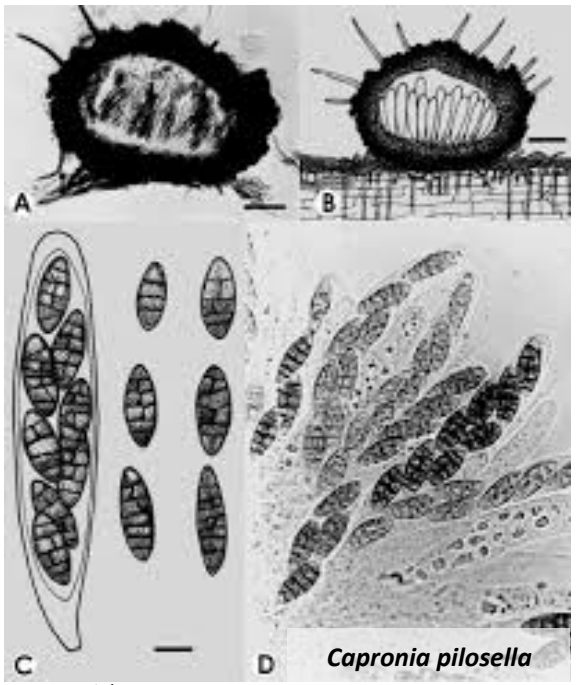
Results: Most frequent lichen-inhabiting fungi

ID	Reads	Lichen samples	Identlty (%)	GenBank#	Match	Isolation source
OTU_037	1219	18	99	KF823589	uncultured *Fibulobasidium*	dead wood of *Fagus sylvatica*
OTU_230	189	14	98	EU139148	*Capronia sp.* 94006a	*Umbilicaria mammulata*
OTU_043	1151	13	91	KF296787	uncultured *Chaetothyriales*	soil
OTU_119	349	11	99	AM999722	uncultured *Tremellales*	bryophyte
OTU_091	384	9	93	GU122904	uncultured *Capnodiales*	wood chips
OTU_133	184	9	99	EF521252	uncultured *Chaetothyriales*	soil in spruce forest
OTU_225	117	7	99	KJ867418	*Cladosporium arthropodii*	*Umbellularia californica* leaves
OTU_239	114	7	98	KC965446	uncultured *Hypocreales*	soil
OTU_273	107	7	86	KF225852	uncultured *Cryptococcus*	forest soil
OTU_072	405	6	99	GU187504	*Athelia arachnoidea*	*Populus sp.* leaves



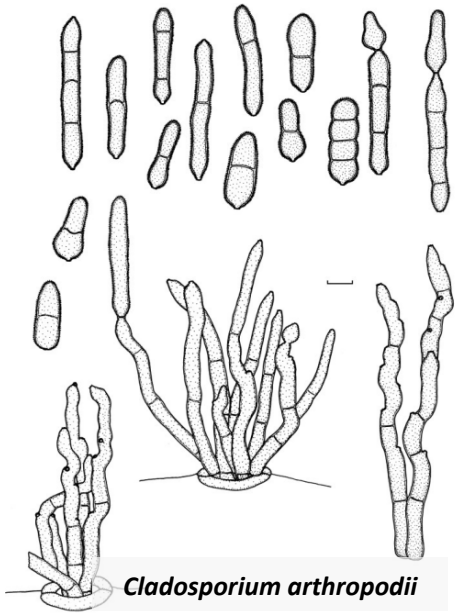
Fibulobasidium inconspicuum

Bandoni 1979



Capronia pilosella

W.H. Hsieh



Cladosporium arthropodii

Bensch et al. 2012

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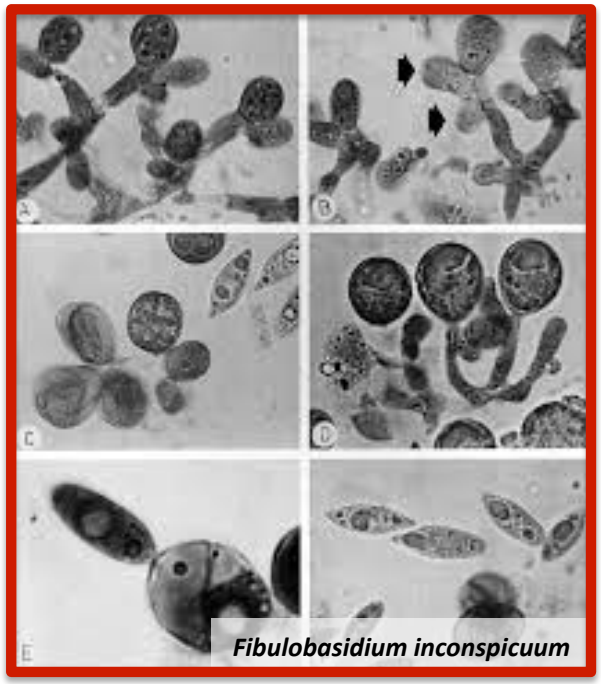

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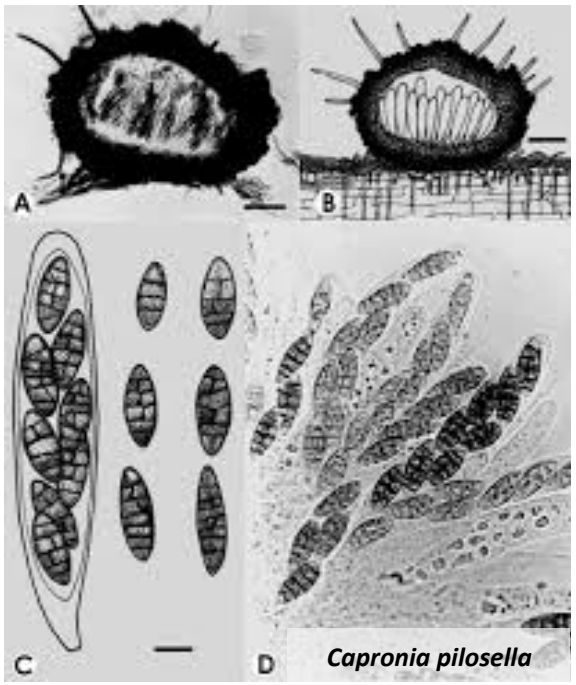
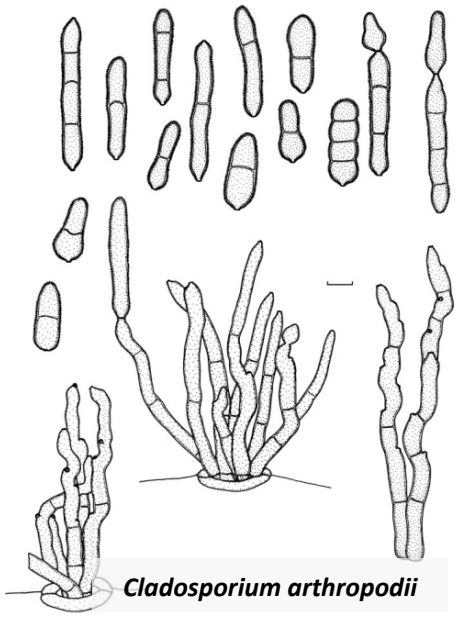


Capronia pilosella



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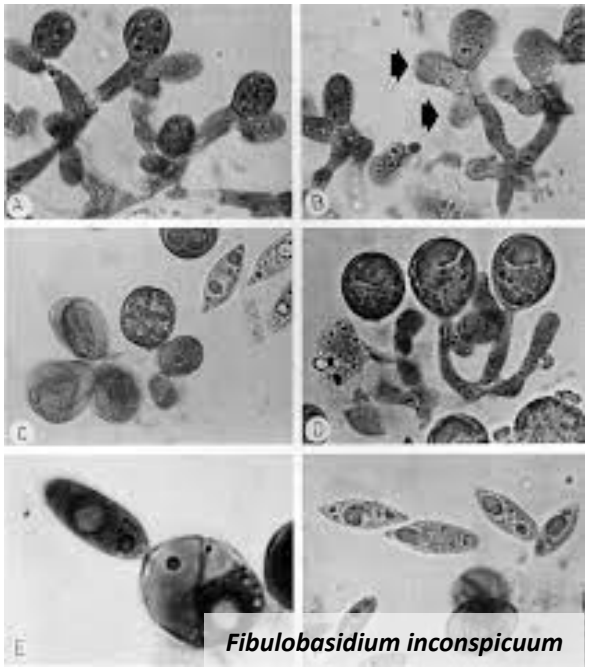

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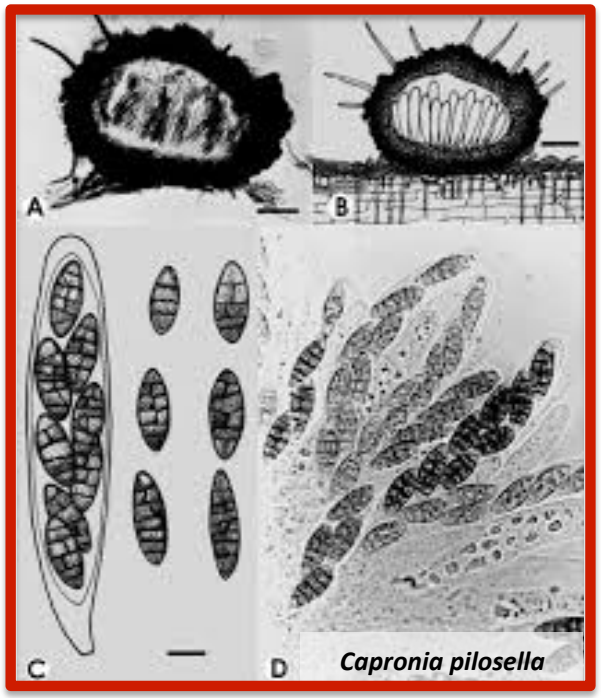

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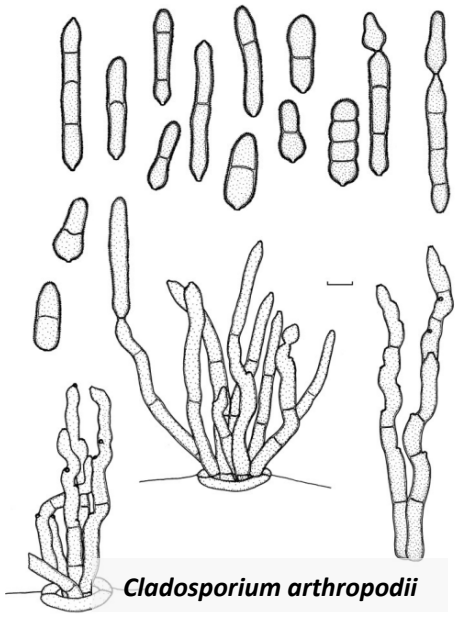

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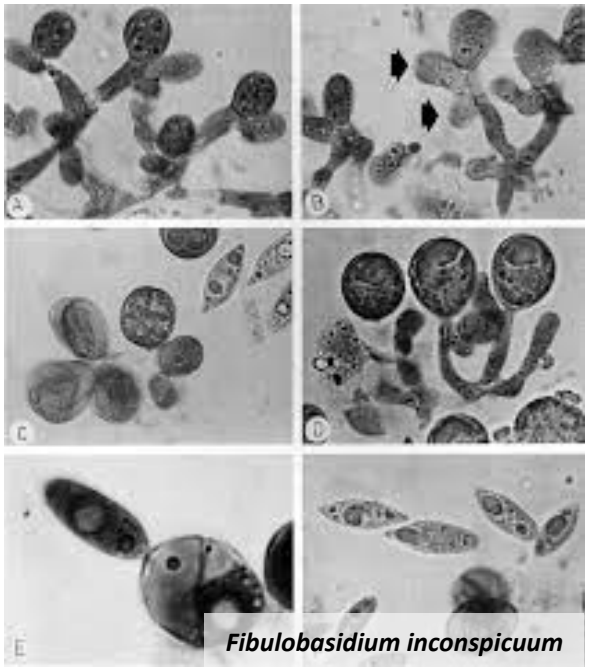
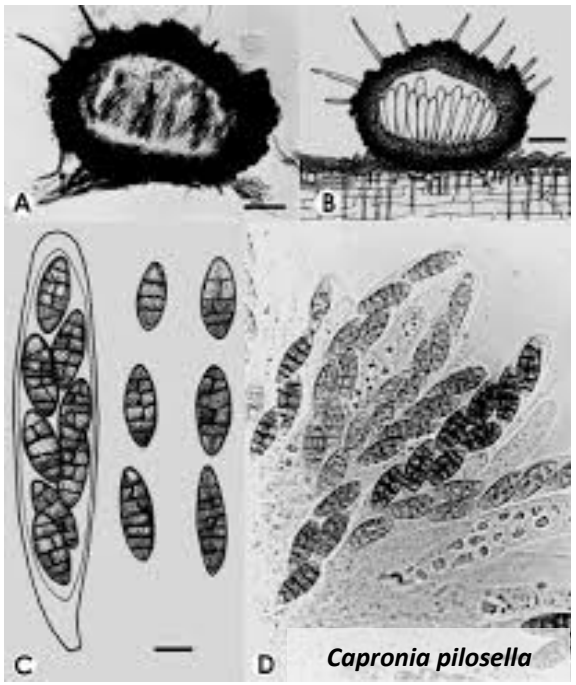
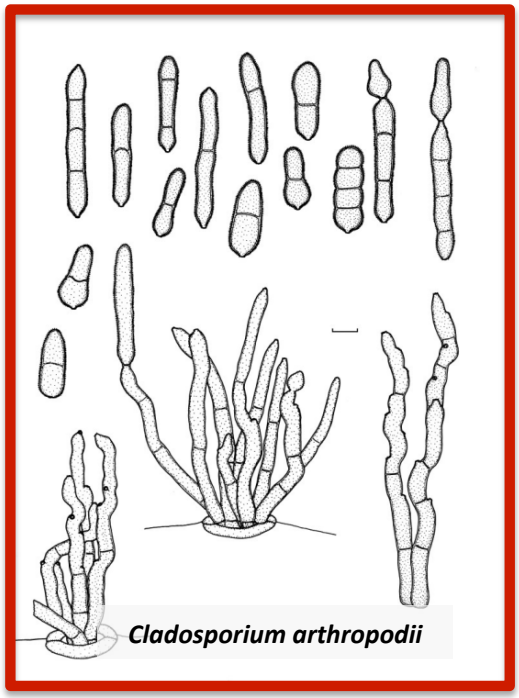
OTU_043	1151	13	91	KF296787	uncultured *Chaetothyriales*	soil
OTU_119	349	11	99	AM999722	uncultured *Tremellales*	bryophyte
OTU_091	384	9	93	GU122904	uncultured *Capnodiales*	wood chips
OTU_133	184	9	99	EF521252	uncultured *Chaetothyriales*	soil in spruce forest
OTU_225	117	**7**	99	KJ867418	***Cladosporium arthropodii***	*Umbellularia californica* leaves
OTU_239	114	7	98	KC965446	uncultured *Hypocreales*	soil
OTU_273	107	7	86	KF225852	uncultured *Cryptococcus*	forest soil
OTU_072	405	6	99	GU187504	*Athelia arachnoidea*	*Populus sp.* leaves



Fibulobasidium inconspicuum



Capronia pilosella



Cladosporium arthropodii