Netherlands Food and Consumer
Product Safety Authority
Ministry of Agriculture, Fisheries,
Food Security and Nature

MUSTI:
Mycological Universal Strain Typing & Indentification

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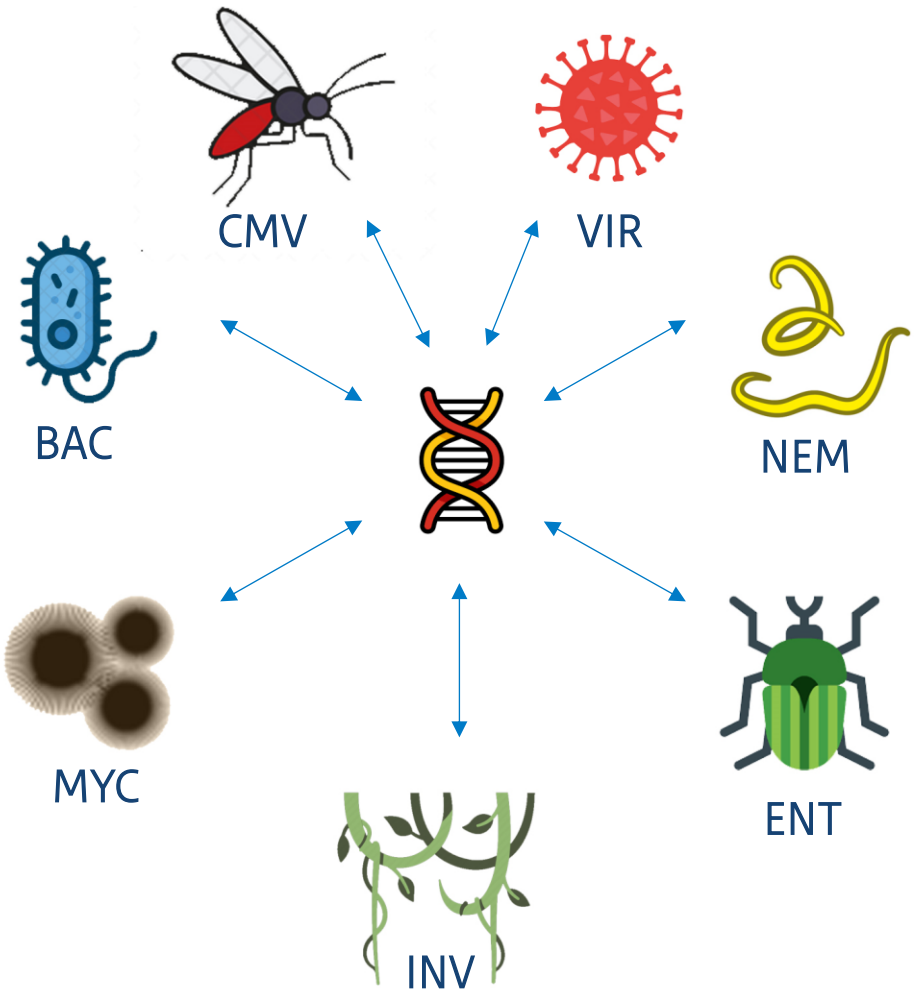
April 2026 – EPPO conference on Diagnostics of Plant Pests

Background

- The molecular biological group supports organism specific groups in diagnostics of plant pests and vectors
- For mycology, identification of fungal strains is supported with sequencing analysis
- Traditionally, PCR sequencing of ITS barcode with one or more extra loci

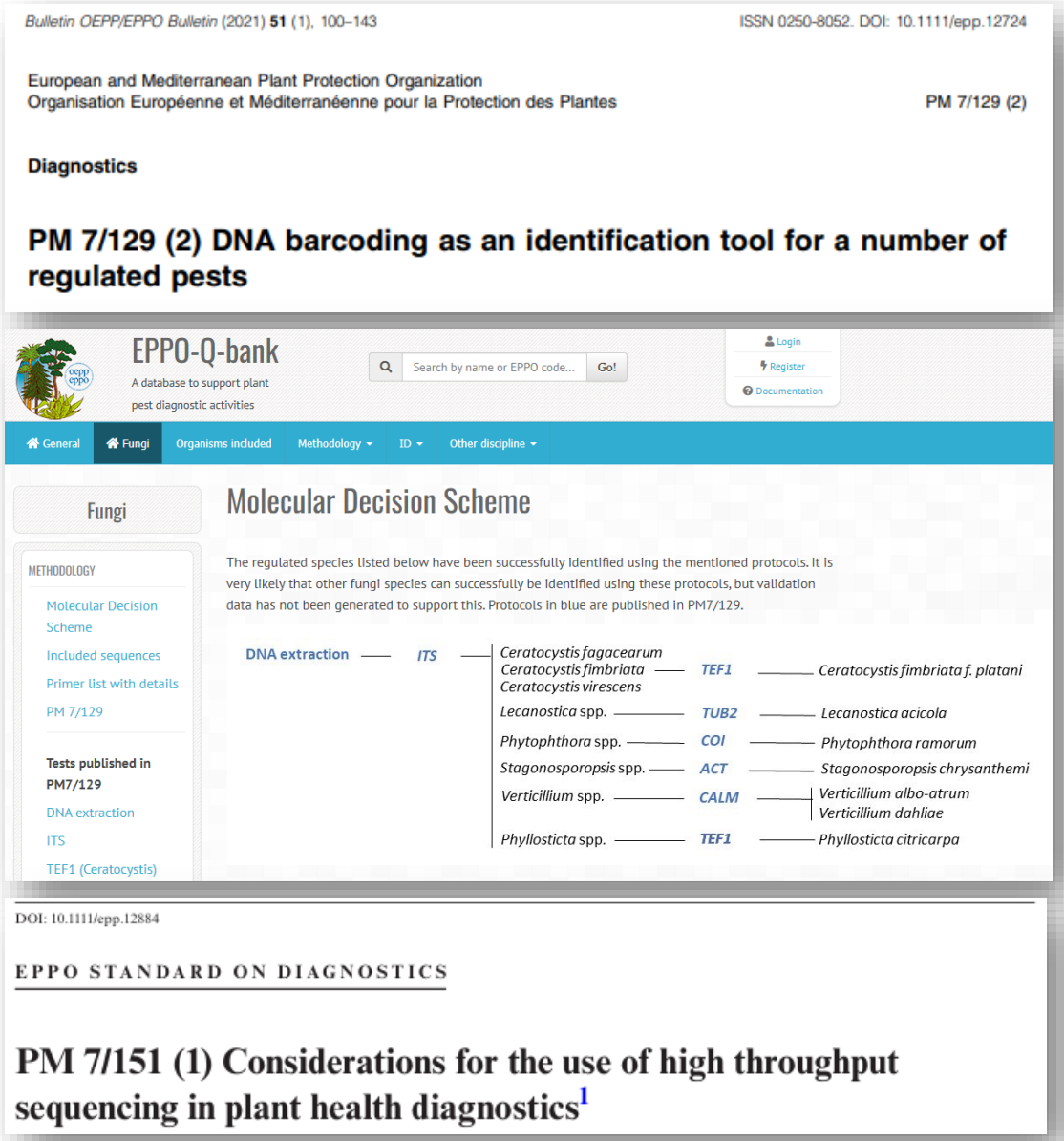
Challenges for regulated fungi:

- The ITS barcode often lacks resolution
- Additional loci are only limited standardized
- Often primers for additional loci are not implemented or known



Project aims

- Development of a generic high throughput sequencing (HTS) test for culturable fungi
- Extract frequently used barcodes from the whole genome (rDNA, EF1a, RPB2, ACT, CALM, TUB)
- Broad implementation for culturable regulated fungi
- Replace Sanger sequencing for identification purposes



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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/129 (2)

Diagnostics

PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests

EPPO-Q-bank
A database to support plant pest diagnostic activities

Search by name or EPPO code... Go!

Login
Register
Documentation

General | Fungi | Organisms included | Methodology | ID | Other discipline

Fungi

METHODOLOGY
- Molecular Decision Scheme
- Included sequences
- Primer list with details
- PM 7/129

Tests published in PM7/129
- DNA extraction
- ITS
- TEF1 (Ceratocystis)

Molecular Decision Scheme

The regulated species listed below have been successfully identified using the mentioned protocols. It is very likely that other fungi species can successfully be identified using these protocols, but validation data has not been generated to support this. Protocols in blue are published in PM7/129.

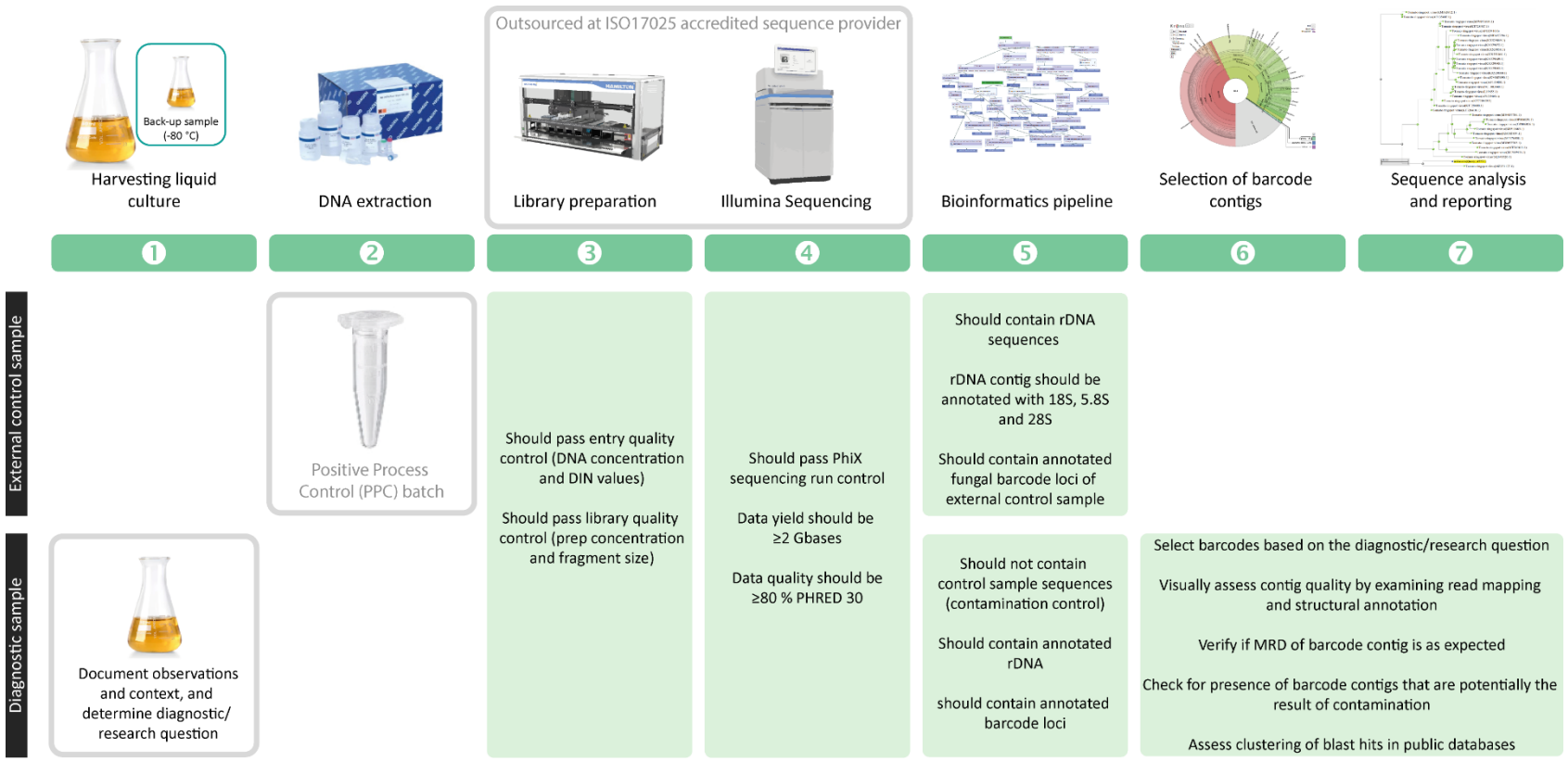
DNA extraction	ITS	*Ceratocystis fagacearum* *Ceratocystis fimbriata* *Ceratocystis virescens*	TEF1 — *Ceratocystis fimbriata f. platani*
		Lecanostica spp.	TUB2 — *Lecanostica acicola*
		Phytophthora spp.	COI — *Phytophthora ramorum*
		Stagonosporopsis spp.	ACT — *Stagonosporopsis chrysanthemi*
		Verticillium spp.	CALM — *Verticillium albo-atrum* *Verticillium dahliae*
		Phyllosticta spp.	TEF1 — *Phyllosticta citricarpa*

DOI: 10.1111/epp.12884

EPPO STANDARD ON DIAGNOSTICS

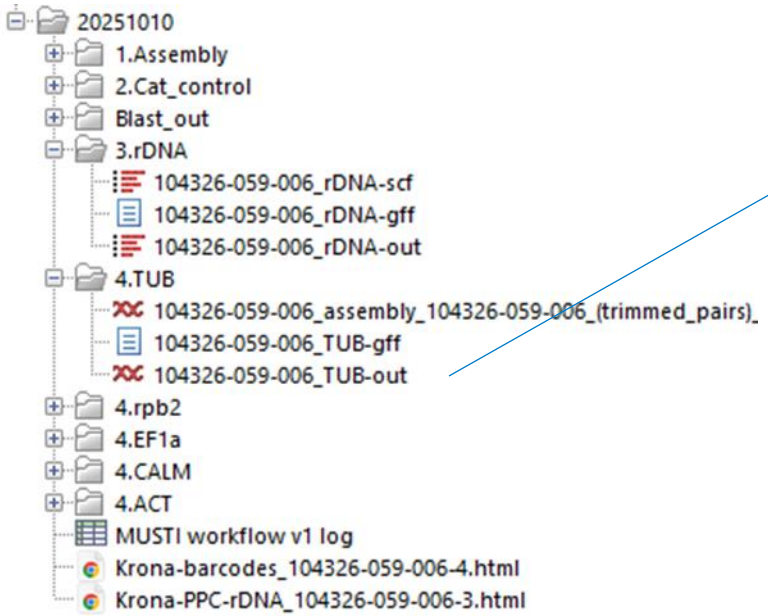
PM 7/151 (1) Considerations for the use of high throughput sequencing in plant health diagnostics[1]

The test – from sample to sequence report



- Liquid monosporic or hyphal tip cultures serve as input
- *Fusarium venenatum* serves as external control sample (Alien control)
- Samples and alien control are used to monitor positive and negative control steps
- Visual assessment of consensus sequences and clustering prior to reporting

Bio-informatic output



- Whole genome assembly with contigs containing barcodes
- In-silico verification of barcodes with oligo's
- Extraction for downstream analysis (PM7/129)

Output is recorded on a standardized form

1

Title	Analysis of Illumina sequence data in support of fungal species identification			
code	F-MOL-137-001	version 01	08-09-2025	p. 1 of 5

Date: 12-9-2025 1st assessor: Bart van de Vossenberg 2nd assessor: Tijs van den Bosch

1. PPC control used:
2. *n* reads mapped to cat mtDNA: 32 blast based ID: no hits contamination control: ☒ Pass ☐ Fail: follow-up:

T.J.M. van den Bosch (Tijs) Digitally signed by T.J.M. van den Bosch (Tijs) Date: 2025.09.22 16:58:36 +02'00'

Table 1. Illumina sequence data information and the obtained barcodes for analysis.

1	LIMS or collection ID	CPC18535 CPC18535					
2	Sequence data generated by	☒ In-house ☐ GenomeScan					
3	Batch + sequence ID	210608_VH00147_12					
4	*n* reads: average length	After read trimming: 32240498 reads; 117.41 bp (3.79 Gb)					
5a	*De novo* assembled scaffolds	192					
5b	Optional remarks *de novo* assembly (e.g. ~median ARC; assembly size)	Estimated median ARC: 25.42; Assembly size = 138 Mb					
6	MUSTI workflow version	V1					
7	Target	rDNA	EF1a	RPB2	CALM	TUB2	ACT
7a	*n* (target) blast hits	2	1	2	1	1	1
7b	Barcode recovered	☒ yes ☐ no	☒ yes ☐ no	☒ yes ☐ no	☒ yes ☐ no	☒ yes ☐ no	☒ yes ☐ no
7c	Primers used (Terminal primers that define the extracted barcode. Frequently observed terminal primers are listed first in this table)	☒ ITS4; ☒ ITS5 ☐ ITS1	☒ EFCF1; ☒ EFCF2 ☐ EF1-728F; ☐ TEF1-983F; ☐ EF1-986R; ☐ EF-2;	☒ fRPB2-5F; ☒ fRPB2-7R ☐ fRPB2-414R	☒ CL1C; ☒ CL2C ☐ CAL-228F; ☐ CL1 ☐ CAL-235F; ☐ CAL-737R; ☐ CL2A; ☐ CAL2Rd;	☒ T1; ☐ Bt-2b ☐ TUB4Rd; ☐ TUB2Fd; ☒ T2; ☐ Btub_F1	☒ ACT2Rd; ☒ ACT-512F ☐ ACT-783R
7d	Barcode length	655	809	1178	695	593	613
7e	Remarks mapping and assembly barcodes (optional)						

Note that when more than 1 barcode sequence is obtained per locus, the sample could have contained multiple specimens and species. For this reason it is important to analyze the datasets in the context of the batch in which they were generated. When the blast-based putative identity clearly indicates these are environmental contaminants (e.g. human), these do not need to be further analyzed.

2

Title	Analysis of Illumina sequence data in support of fungal species identification			
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Table 2. Information regarding the analysis and consulted (on-line) resources

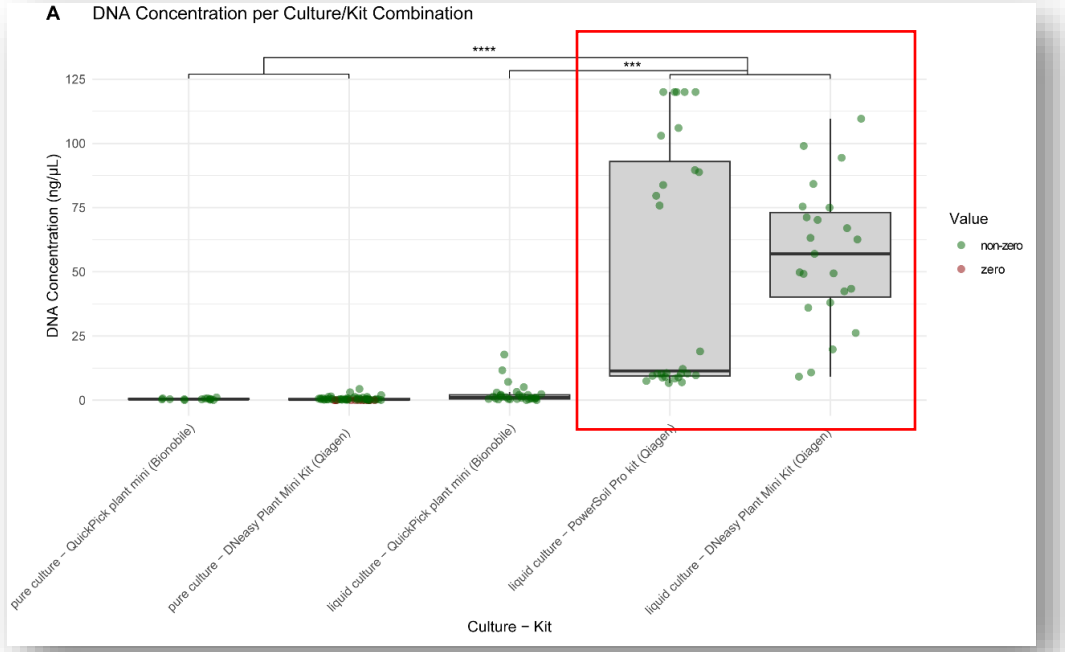
Source	Analysis parameters	Settings	Explanation, reference to analysis results and conclusions per database[$]
NCBI	Database consulted	☒ Core_nt ☐ nt/nr collection ☐ other#	Figure 1. FME clustering of the rDNA blast hits results in clustering with several Elsinoë species
	Blast type	☒ Megablast ☐ Discont. Megablast ☐ blastn	Figure 2-4. FME clustering of the ACT/CALM/EF1a blast hits do not result in meaningful clustering
	Tree method	☒ Fast Minimum Evolution (FME) ☐ NJ	Figure 5. FME clustering of the rpb2 blast hit results in clustering with the Elsinoë citricola type species
	Organism*	☒ not used ☐ used#	Figure 6. FME clustering of the TUB blast hits does not result in meaningful clustering
	Exclude taxon*	☒ not used ☐ used#	
	Exclude uncultured*	☒ not used ☐ used	
	Types only*	☒ not used ☐ used	
BOLD	Database consulted	☐ Fungi Library	not used
	Operating Mode	☐ Rapid Species ☐ Genus and Species ☐ Exhaustive (standard)	
Q-bank	Analysis method	☐ Single locus ☐ Multi locus#	not used
	Settings adjusted	☐ no ☐ yes#	
	Tree method	When applicable#	
Data NVWA	Dataset used	Blast file:	not used

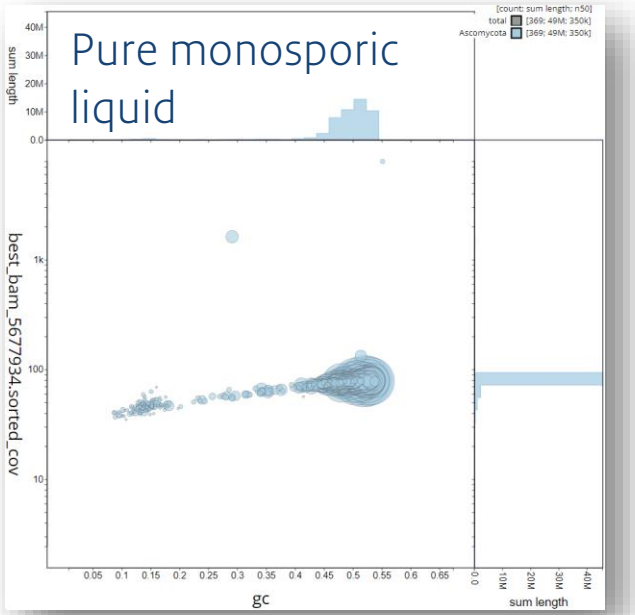
Provide details in the last column of the table; * Optional; $ e.g. number of nucleotides in analysis, % overlap, % similarity with 1st and/or specific match, E-value, clustering with taxon Z

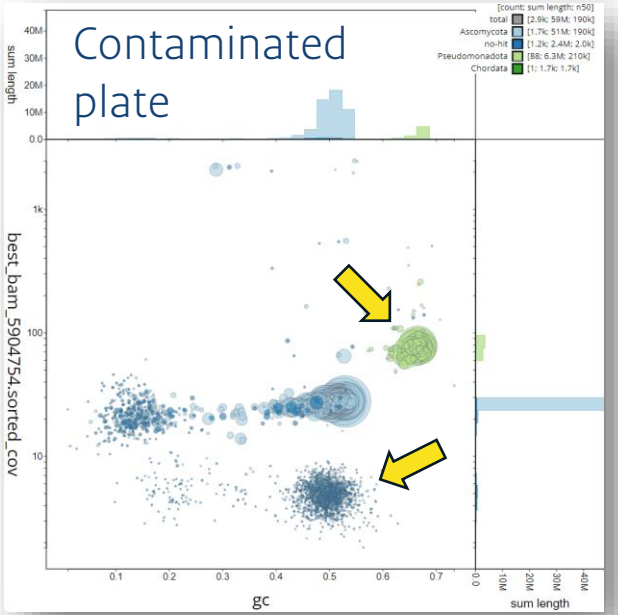
- Assembly and barcode statistics and analysis of obtained barcodes
- Four-eyes principle

Liquid cultures outperform cultures from plates

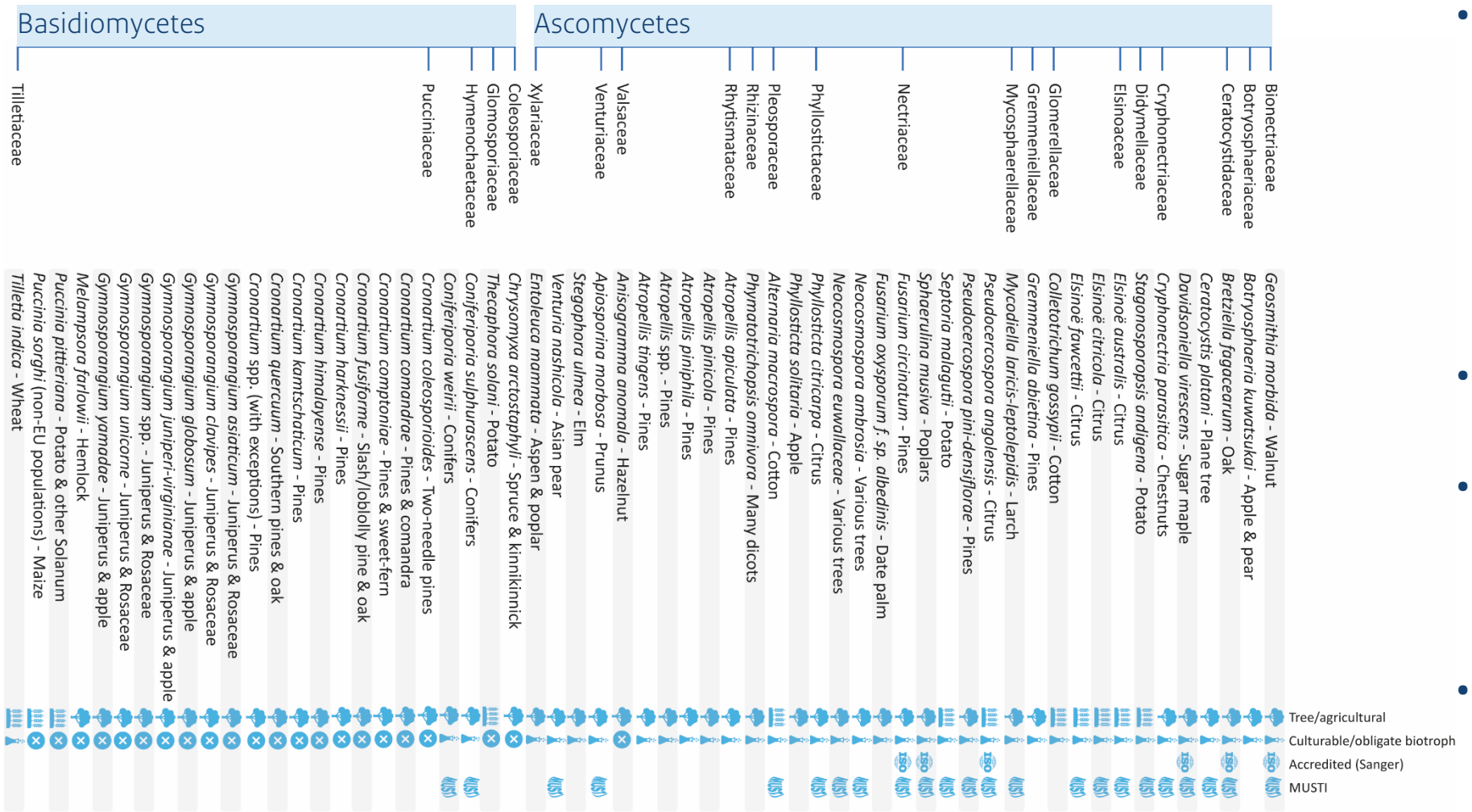
- Higher DNA concentration facilitates more robust library preparation and sequencing
- Monosporic or hyphal tip ensures purity of fungal cultures making data interpretation easier





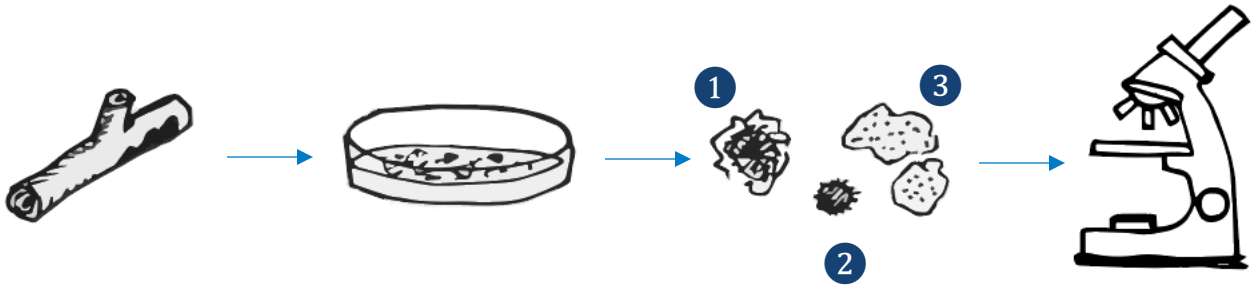


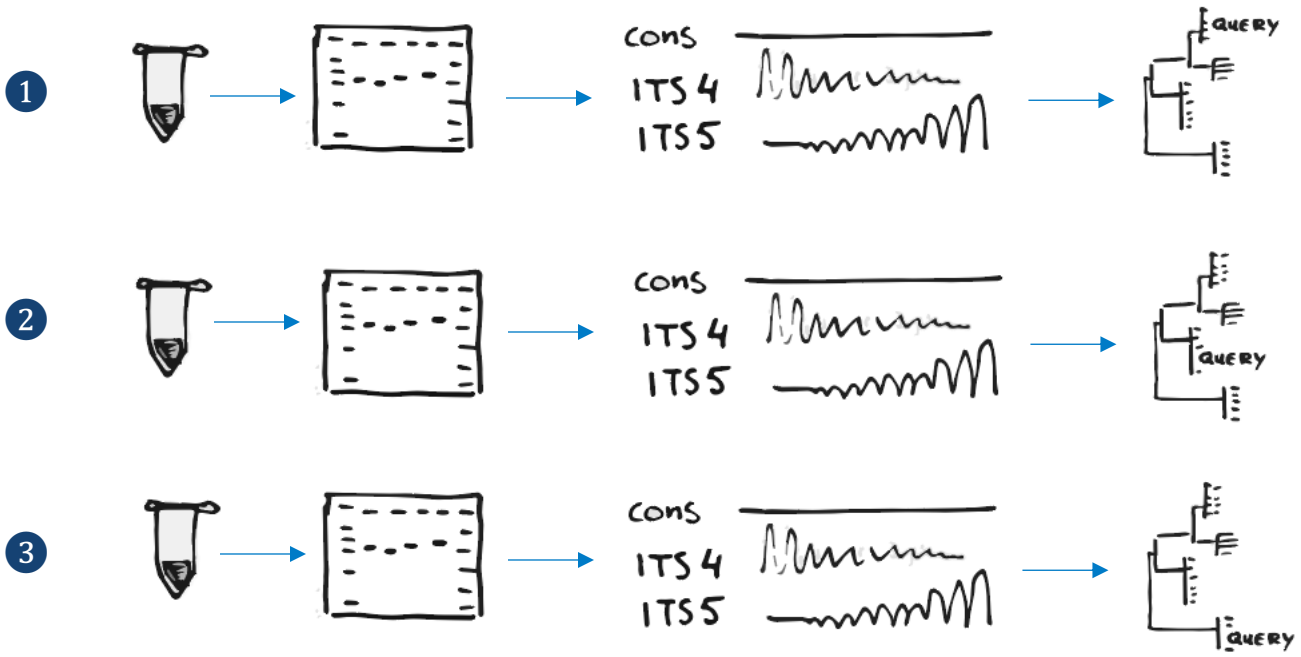
Validation according to PM7/98(6)

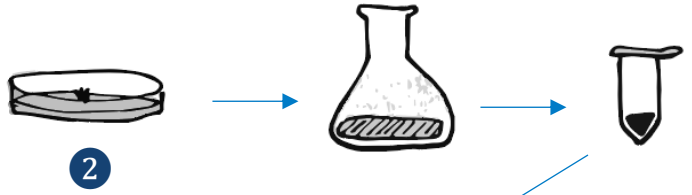


- Scope: identification of axenically cultured fungal regulated species using selected barcodes obtained with High Throughput Sequencing
- LOD = minimal 108.1 Mb data and 14x MRD
- Specificity: 37 regulated culturable fungi, 23 could be obtained and tested. 100% conformity.
- 100% repeatable results

MUSTI in the context of plant health diagnostics







- Sanger sequencing (ITS) still has a valuable place in the diagnostic process

Conclusions and Outlooks

- MUSTI overcomes challenges presented by traditional Sanger sequencing for culturable regulated fungi
- MUSTI was demonstrated fit for purpose for all regulated fungi tested (23 of 37 fungal species)
- Obtaining additional fungal species and test verification is planned
- We will explore using the entire fungal genome (ANI based) for species identification. First experiences with *Neocosmospora* spp. and *Elsinoë* spp. are very promising.

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Thank you for your attention

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