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LABORATORY METHODS

Analytical Method

Identification of Culturable Moulds

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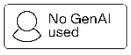
Disclosure regarding the use of Generative Artificial Intelligence 

VERSION HISTORY

DESCRIPTION OF CHANGES
This version has been reformatted using the new analytical method template. It provides an update on the analytical techniques used to identify culturable moulds in the IRSST laboratory, namely the addition of MALDI-TOF mass spectrometry and Sanger sequencing targeting different genetic loci.

TARGET MICROBIAL AGENTS
Culturable moulds

APPLICATION

This method is used to identify culturable moulds cultivated from air, surface, solid or liquid samples at the genus and species levels. Mould strains are isolated in pure culture, after which different techniques can be used: observation of macroscopic and microscopic features of colonies and comparison to reference identification keys, mass spectrometry using MALDI-TOF technology and Sanger sequencing targeting different genetic loci. Identifications obtained by one, several or all of these techniques are then interpreted to produce a final identification.

LIMITATIONS AND INTERFERENCES

The performance of this method may be affected by a number of factors:

- Presence of growth conditions suitable for mould development;
- Presence of confluent growth or invasive microorganisms on culture media, which may hinder pure culture isolation;
- Maintenance of strain culturability during pure culture isolation;
- Maintenance of the expected phenotypic characteristics of the strain to be identified;
- Ability of the strain to sporulate on the type of medium used, since sporulation is necessary for identification by observing the macroscopic and microscopic features and may influence MALDI-TOF mass spectrometry results;
- Presence of the target mould in the databases used, and validity of the entries in those databases;
- A change in nomenclature that is not reflected in the identification keys in the manuals consulted and/or the databases searched.

REAGENTS AND MATERIAL

Isolation in pure culture and observation of macroscopic and microscopic features

- Malt extract agar and other culture media as needed
- Mycological culture tools
- Lactophenol blue dye (or equivalent)
- Reference manuals (see Appendix 1)

MALDI-TOF mass spectrometry

- Sabouraud agar
- Absolute ethanol
- Formic acid 70%
- Acetonitrile
- VITEK® MS-DS slides and VITEK® MS-CHCA matrix

Sanger sequencing

- Quick-DNA™ DNA isolation kit: Fungal/Bacterial MiniPrep (D6005, Zymo Research) or PrepMan™ Ultra Sample Preparation Reagent (4318930, Applied Biosystems) (or equivalent)
- Reagents and materials for PCR (polymerase chain reaction) amplification and cleaning of amplicons (or equivalent)
  - Phusion HF 5X buffer (F-518, Thermo Fisher)
  - dNTPs mix 10 mM (R0192, Thermo Fisher)
  - Pairs of primers 25 µM (see Table 3)
  - Phusion polymerase (F-530L, Thermo Fisher)
  - MinElute 96 UF PCR purification plates (FG3829, QIAGEN)
- Reagents and materials for gel electrophoresis of amplicons
- QIAquick PCR & Gel Cleanup Kit (28704, QIAGEN) (or equivalent) (optional)
- Reagents and materials for product sequencing, precipitation/ purification reactions (or equivalent)
  - BigDye v3.1 5X sequencing buffer (4336697, Thermo Fisher)
  - BigDye Terminator v3.1 Cycle sequencing kit (4336917, Thermo Fisher)
  - Primers 3 µM
  - EDTA 125 mM
  - Sodium acetate 3M
  - Absolute ethanol
  - Ethanol 70%
  - Hi-Di Formamide (441457, Applied Biosystems)

EQUIPMENT

Isolation in pure culture and observation of macroscopic and microscopic features

- Class II biosafety cabinet
- Incubator (25°C ± 2°C or other temperature as needed)
- UV lamp (optional)
- Stereomicroscope with up to 12X magnification (optional)
- Phase-contrast transmitted-light microscope with up to 1000X magnification

MALDI-TOF mass spectrometry

- Microcentrifuge
- VITEK® MS MALDI-TOF microbial identification system from bioMérieux (including VITEK MS V3.2 knowledge base or a subsequent version)

Sanger sequencing

- T100 thermocycler from Biorad (or equivalent)
- SeqStudio Genetic Analyzer sequencer from Applied Biosystems
- SeqMan Ultra sequence analysis software from DNAStar (or equivalent)

PREPARATION

Step 1	**Isolation in pure culture** a) Under the biosafety cabinet, subculture, by central inoculation on malt extract agar, the specified number (1, 2, 3 or all) of distinct colonial morphotypes observed on the initial agar. b) Incubate at 25°C ± 2°C for 3 to 14 days, until the colony is well developed and/or sporulation is observed. If necessary, sporulation may be stimulated by exposure to light or the UV lamp. c) Confirm the purity of the isolated colony and the correspondence with the original colony on the initial agar before proceeding to the next steps. Note: Other kinds of culture media and/or different incubation temperatures may be used for specific cases. When the isolation steps result in a loss of viability or a mixed culture, the result is reported as "non-isolable."
Step 2	**Observation of macroscopic and microscopic features (technique 1.a) and 1.b))** a) For each isolate, observe and note the macroscopic features of colonies (size, colour, texture, media pigmentation, etc.). If necessary, use a stereomicroscope for certain more detailed observations, such as the arrangement and conformation in 3 dimensions. b) Prepare a wet mount with a drop of lactophenol blue dye and verify the microscopic features (size, texture, shape of conidia, conidiophores, hyphae, etc.) under the microscope. c) Using the identification keys in the reference manuals, identify the genus of the mould. When the observations are clear, it is also possible to suggest one or more species. Note: This technique generally allows identification at the genus level but rarely the species level. It is also possible that the observed features do not allow the identification of the genus or that the genus is unknown. In that case, it is desirable to continue the identification using the analytical techniques in step 3.
Step 3	Continue the identification by using one (or both) of the following complementary analytical techniques: • **Mycological identification by MALDI-TOF mass spectrometry (technique 2)** • **Mycological identification by Sanger sequencing (technique 3)** Note: Typically, the analytical techniques are applied in the order shown in the diagram in Figure 1. However, it is possible to combine these techniques or to opt for only one of them, depending on the context.
Step 4	**Final interpretation and reporting of results** The results obtained by the various techniques used are interpreted to produce a final identification at the genus or species level. For identification at species level, it may be relevant to validate the final result by comparing the macroscopic and microscopic features of the reported identification with those initially observed. In case of doubt regarding the identified species, it is preferable to report only the genus, or if applicable, the complex or section level, or to indicate the species that cannot be discriminated with the techniques used. When even the genus of the strain cannot be identified, the result "not identifiable" is reported.

A diagram setting out these steps is shown in Figure 1.

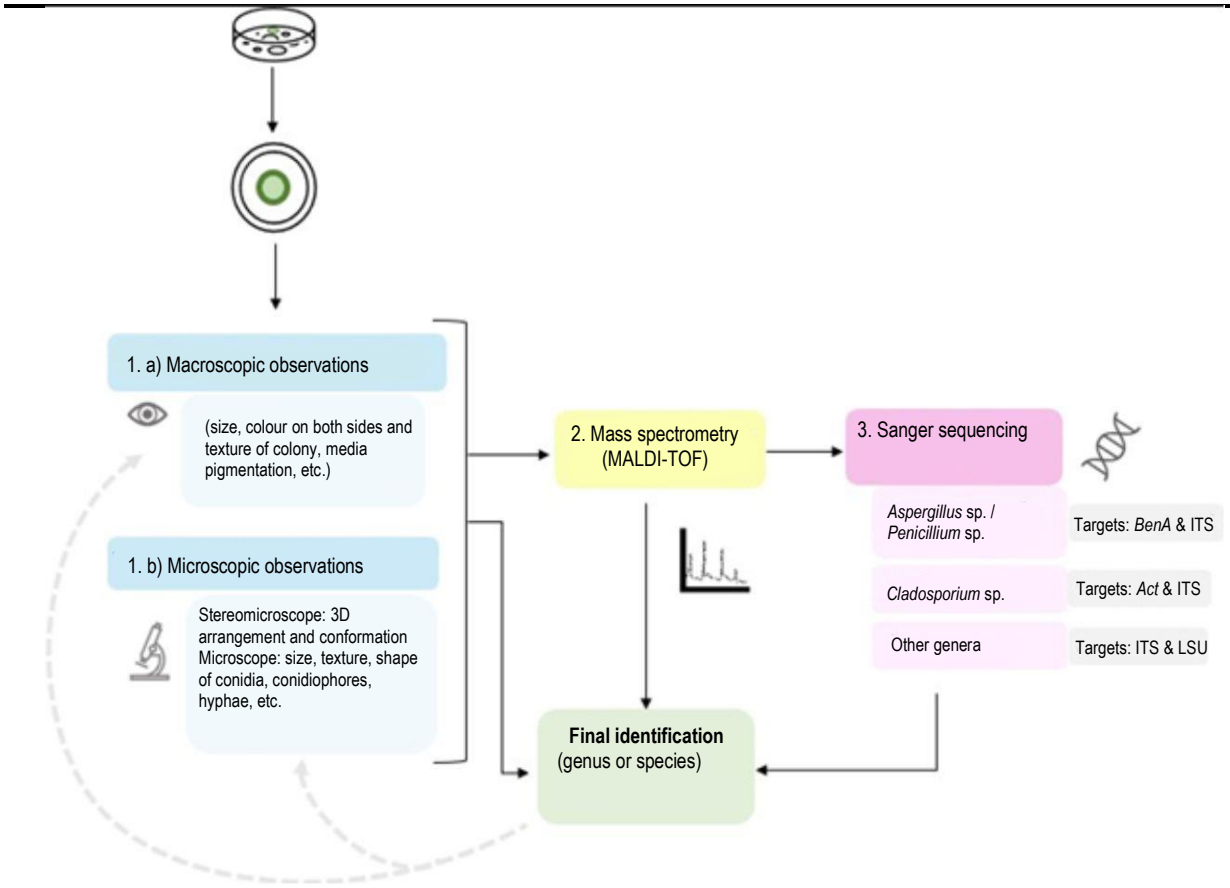


Figure 1. Diagram of the identification process for culturable moulds.

ANALYTICAL TECHNIQUE 2 – MYCOLOGICAL IDENTIFICATION BY MALDI-TOF MASS SPECTROMETRY

Principle

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF) mass spectrometry can be used to identify microorganisms (bacteria, yeasts, moulds) by analyzing their protein profiles. For mycological identification, cells are harvested and then extracted in ethanol, formic acid 70% and acetonitrile. The extract obtained is deposited on a spot (target) on the slide, then crystallized by adding a matrix. This deposit is then targeted by the instrument's laser beam. The molecules of the microorganism are then desorbed and ionized so they can be separated by size through acceleration in an electromagnetic field. The instrument measures the mass-to-charge ratios of the molecules by determining the time it takes for them to travel the length of the flight tube. The protein profile (spectrum) obtained is then compared to a database of reference spectra, allowing the instrument to return an identification of the microorganism analyzed.

System and methodology

The bioMérieux VITEK® MALDI-TOF mass spectrometry system is used. Each disposable slide has a unique bar code and comes with three sections of 16 sample spots, for a total of 48 sample spots per slide. The instrument's holder can accommodate a maximum of 4 slides at a time, so a maximum of 192 analyses can be done simultaneously.
The protocol for the extraction and preparation of spots on a slide is a version adapted from the procedure used by BCCM-IHEM.

1. Under the biosafety cabinet, the strains to be identified are subcultured on Sabouraud agar and incubated at 25°C (or a different temperature if necessary) for 3 days or until the colony has developed sufficiently and/or sporulation has started.
2. The mycelium is harvested, taking care to avoid picking up the agar, then transferred into a microtube containing a volume of water, to which 3 volumes of absolute ethanol are added.
3. After vortexing, the pellet is compacted by centrifugation and dried.
4. A volume of 50 µL of formic acid 70% is added to the pellet, then vortexed.
5. After 5 minutes, step 4 is repeated with acetonitrile.
6. After 5 minutes, the extract is clarified by centrifugation.
7. A volume of 1 µL of supernatant is deposited on a target, then dried.
   a) Generally, each extract is deposited on 4 replicas (4 targets) on the slide, to ensure the repeatability of the ID results.
8. A volume of 1 µL of MS-CHCA matrix is added, then dried.
9. The following controls are added to each slide analyzed: a slide negative control (water + matrix only), an extraction negative control (extraction reagents only) and a positive control of a reference strain (e.g., *Aspergillus brasiliensis*).
10. The VITEK® MS IVD database is automatically queried when the system is used. The knowledge base in effect at the time of publication of this method is version 3.2.
11. The spectral files generated by the VITEK® MS are used to query an external database, the Mass Spectrometry Identification (MSI) platform (MSI V2.0). This platform uses its own interpretive criteria to identify at the genus or species level.

Since each extract is deposited in 4 replicas (step 7), we obtain 4 identifications per isolate and per database queried. The final identification generally retained for each database is the one that represents the majority of results obtained (at least 3 out of 4).

Comments:
Please refer to the manufacturer's instructions for specific directions on the use of the bioMérieux VITEK® MS system.

VALIDATION

The parameters normally assessed during methodology development (limit of detection and limit of quantification, precision, accuracy, recovery and analytical uncertainty) do not apply to this method. Thus, a summary of ID results obtained based on reference strains is presented in Table 1.

Table 1. Validation results: Mycological identification by VITEK MS coupled with MSI V2 database

Genus/species analyzed (1 strain per species, unless specified in parentheses)	No ID	Correct genus only	Species level		Comments
			Correct	Incorrect	
***Aspergillus* sp.**					
11 strains, 9 species: *A. affinis* *A. brasiliensis* (2) *A. flavus* [a] *A. fumigatus* [b] *A. niger* [b] *A. oryzae* [a] *A. sclerotiorum* (2) *A. sydowii* *A. versicolor*	2/11	2/11 [b]	7/11 [a]	–	[a] Although recorded as correctly identified at the species level, the system cannot discriminate between *A. flavus* and *A. oryzae*. [b] Two identifications obtained at the genus level with MSI were correctly identified at the species or complex level with the IVD database.
***Penicillium* sp.**					
10 strains, 8 species: *P. brevicompactum* [c] *P. chrysogenum* [c] *P. digitatum* *P. expansum* *P. glabrum* *P. lividum* *P. purpurascens* (2) *P. restrictum* (2)	–	4/10 [c]	6/10	–	[c] Two identifications obtained at the genus level with MSI were correctly identified at the species or complex level with the IVD database.
***Cladosporium* sp.**					
5 strains, 5 species: *C. cladosporioides* [d] *C. halotolerans* *C. parahalotolerans* [e] *C. ramotenellum* *C. sphaerospermum*	–	1/5 [e]	3/5	1/5 [d]	[d] The species *C. cladosporioides* was incorrectly identified as *C. westerdijkiae;* consider limiting the ID to the *Cladosporium cladosporioides* complex, which was correctly identified with the IVD database. [e] The system could not identify the species *C. parahalotolerans* (missing from the MSI V2.0. database)
Other genera					
17 strains from 12 genera: *Alternaria alternata* [g] *Alternaria chartarum* *Beauveria bassiana* [g] *Cephalotrichum microsporum* *Fusarium graminearum* [f] *Fusarium sp.* *Marquandomyces marquandii* *Paecilomyces variotii* *Phoma glomerata* *Pleurostoma richardsiae* *Sarocladium strictum* *Scopulariopsis brevicaulis* *Scopulariopsis candida* *Stachybotrys chartarum* *Trichoderma harzianum* *Trichoderma viride* *Wallemia sebi*	1/17	5/17 [g]	10/17	1/17 [f]	[f] The species *Fusarium graminearum* was incorrectly identified as *Fusarium paeoniae*. [g] Two identifications obtained only at the genus level with MSI were correctly identified at the species level with the IVD database.
TOTAL – all genera	**3/43 (7.0%)**	**12/43 (27.9%)**	**26/43 (60.5%)**	**2/43 (4.7%)**	

ANALYTICAL TECHNIQUE 3 – SANGER SEQUENCING

Principle

The first step in Sanger sequencing is extraction and purification of the DNA of the microorganism to be identified. A specific DNA segment, the molecular target, is then amplified by PCR. The target segment is subsequently denatured to separate the two strands of the double-stranded DNA. A polymerization reaction is carried out with sequencing primers on both denatured strands using a DNA polymerase and the chain-terminating nucleotides dNTPs (deoxynucleotide triphosphates) and ddNTPs (dideoxyribonucleoside triphosphates). These chain-terminating nucleotides, each labeled with a unique fluorescent label, cannot form phosphodiester bonds with the next nucleotide in the chain. When a ddNTP is randomly incorporated in the sequence of a newly synthesized strand, the polymerization terminates. This generates a collection of DNA fragments of varying lengths, each ending with a fluorescently labeled ddNTP. The sequencer automatically separates the amplified DNA fragments by size using electrophoresis and runs them through a fluorescence detector to identify the terminal nucleotide of each fragment. The sequencer generates a chromatogram that can be used to reconstruct the DNA sequence of the initially amplified molecular target. This sequence is used to query databases, where it can be compared with the DNA sequences of referenced microorganisms.

SYSTEMS AND METHODOLOGY

1. DNA is extracted using a commercial extraction kit, such as Quick-DNA Fungal/Bacterial MiniPrep or PrepMan™ Ultra Sample Preparation Reagent.
2. Different gene regions are suggested as targets based on the previously identified genus (Table 2).
    a. The primers used for amplification are described in Table 3.
    b. Other gene regions may also be used, depending on specific needs.
3. A thermocycler is used to amplify the targeted gene region, using the following reagents (and final concentrations) in the polymerase buffer (1x): nucleotide mixes (200 µM), pairs of primers (0.5 µM) and polymerase (0.02 U/µL). The thermocycler program steps are set out in Table 4.
4. The size and purity of the amplicons obtained are verified on agarose gel.
5. The amplicons are cleaned with a commercial kit, such as MinElute 96 UF PCR Purification kit from Qiagen.
6. A thermocycler is used to do the sequencing reactions on each DNA strand (sense and antisense) with a commercial kit, such as BigDye™ Terminator v3.1 Cycle, diluted in the sequencing buffer and with only one of the two primers used for the amplification reaction. The thermocycler program steps are set out in Table 4.
7. The labeled DNA strands are then purified, using the ethanol/EDTA/sodium acetate precipitation method described in *DNA Sequencing by Capillary Electrophoresis* (Applied Biosystems, 2009).
8. The capillary electrophoresis step is performed with the SeqStudio sequence analyzer from Applied Biosystems which provides the sequences.
9. The pairs of sequences (sense and antisense) are aligned, corrected and end-trimmed, to produce a single consensus sequence ("contig"), using software such as SeqMan Ultra from DNAStar.
10. The following databases are used to analyze the sequences obtained (Table 2):
    a. NCBI Nucleotide BLAST blastn core nucleotide database (NCBI nt)
    b. NCBI Nucleotide BLAST blastn rRNA/ITS database/ITS from Fungi type and reference material (NCBI ITS)
    c. MycoBank database

Table 2. Suggested targets and databases based on genus of mould identified

Mould genus identified in advance	Targets	Database	References
Aspergillus sp. / *Penicillium* sp.	*BenA* and ITS	NCBI nt or NCBI ITS	Altschul *et al.* (1990); National Center for Biotechnology Information (NCBI) (2025)
Cladosporium sp.	*Act* and ITS		
Other genus	ITS and LSU	NCBI nt or MycoBank	Robert *et al.* (2005)

Table 3. Primers and sequences corresponding to proposed targets

Target	Primer	Sequence (5'-> 3')	References	Expected approximate size of fragment (bp)
ITS	ITS5	GGA AGT AAA AGT CGT AAC AAG G	(CLSI, 2018; Samson et al., 2019; White et al., 1990)	~ 500–600
	ITS4	TCC TCC GCT TAT TGA TAT GC		
LSU	NL1	GCA TAT CAA TAA GCG GAG GAA AAG	(O'Donnell, 1993)	~600
	NL4	GGT CCG TGT TTC AAG ACG G		
BenA	Bt_2a	GGT AAC CAA ATC GGT GCT GCT TTC	(Glass & Donaldson, 1995; Samson et al., 2019)	~ 400–500
	Bt_2b	ACC CTC AGT GTA GTG ACC CTT GGC		
Act	ACT-512F	ATG TGC AAG GCC GGT TTC GC	(Carbone & Kohn, 1999)	~ 200–250
	ACT-783R	TAC GAG TCC TTC TGG CCC AT		

Table 4. Proposed PCR amplification and sequencing reaction programs

PCR amplification			Sequencing reaction		
Step	**Temp. – Dur.**	**Repetition**	**Step**	**Temp. – Dur.**	**Repetition**
Initial denaturation	98 °C – 30 s	–	Initial denaturation	96 °C – 1 min	–
Denaturation	98 °C – 10 s	35 X	Denaturation	96 °C – 10 s	35 X
Hybridization	54 °C – 30 s		Hybridization	50 °C – 5 s	
Elongation	72 °C – 45 s		Elongation	60 °C – 4 min	
Final elongation	72 °C – 10 min	–	–	–	–
Hold	4 °C – ∞	–	Hold	4 °C – ∞	–

Comments:

Please refer to the manufacturer's instructions for specific directions on the use of each commercial kit.

VALIDATION

The parameters typically assessed during method development (limit of detection and limit of quantification, precision, accuracy, recovery and analytical uncertainty) do not apply to this method. Thus, a summary of ID results obtained using reference strains is presented in Table 5.

At present, there is no consensus concerning the acceptability criteria for mycological identification at the genus or species level by sequencing (CLSI, 2018). The values obtained during validation (% of identity and the database with which most IDs were obtained) are provided purposes in Table 5.

Table 5. Validation results: Mycological identification by Sanger sequencing

Genus/species analyzed (1 strain per species unless specified in parentheses)	Target 1			Target 2			Comments
	Genus level only – Correct	Species level		Genus level only – Correct	Species level		
		Correct – Single choice	Several choices or Incorrect		Correct – Single choice	Several choices or Incorrect	
***Aspergillus* sp.**		***BenA***			**ITS**		
16 strains, 15 species: *A. brasiliensis* *A. candidus* *A. flavus* *A. fumigatus* *A. niger* *A. ochraceus* *A. oryzae* *A. ostianus* *A. penicillioides* *A. restrictus* *A. sclerotiorum* (2) [a] *A. sydowii* *A. terreus* *A. unguis* *A. versicolor*	0/15	12/15	3/15 0/15	0/16	10/16	6/16 0/16	[a] A poor-quality sequence of *A. sclerotiorum* for the *BenA* target. General criteria: • *BenA:* > 97% identity; NCBI nt (13/15) • ITS: > 99% identity; NCBI nt (12/16)
Penicillium sp.		***BenA***			**ITS**		
16 strains, 14 species: *P. aurantiogriseum* *P. brevicompactum* *P. chrysogenum* *P. commune* *P. corylophilum* *P. crustosum* *P. digitatum* *P. echinulatum* *P. expansum* *P. glabrum* *P. lividum* *P. purpurascens (2)* *P. restrictum (2)* *P. spinulosum*	0/16	14/16	2/16 0/16	4/16	9/16	3/16 0/16	General criteria: • *BenA:* > 97% identity; NCBI nt (16/16) • ITS: > 99% identity; NCBI nt (14/16)
***Cladosporium* sp.**		***Act***			**ITS**		
9 strains, 7 species: *C. allicinum* [b] *(C. sphaerospermum)* *C. cladosporioides* (2) [c] *C. halotolerans* *C. parahalotolerans* [c] *C. pulvericola* *C. ramotenellum* (2) *C. sphaerospermum*	0/8	6/8	0/8 2/8 [c]	0/9	6/9	3/9 0/9	[b] A poor-quality sequence of *C. allicinum* for the *Act* target. [c] Incorrect species-level identification for a strain of *C. cladosporioides* and *C. parahalotolerans* with *Act*. General criteria: • *Act:* > 96% identity; NCBI nt (8/8) • ITS: > 99% identity; NCBI nt (9/9)
Other genera		**LSU**			**ITS**		
20 strains *Acremonium polychromum* *Alternaria alternata* *Alternaria chartarum* *Alternaria tenuissima* *Ascotricha chartarum* *Beauveria bassiana* *Cadophora melinii* *Epicoccum nigrum* *Fusarium avenaceum* *Fusarium graminearum* *Marquandomyces marquandii* *Paecilomyces variotii* *Paramyrothecium parvum* *Phoma herbarum* [d] *Pleurostoma richardsiae* *Sarocladium strictum* *Scopulariopsis brevicaulis* *Stachybotrys chartarum* *Trichoderma harzianum* [e] *Trichoderma viride*	4/20	11/20	5/20 0/20	3/20	17/20	5/20 0/20	[d] No identification for this strain with the LSU target. General criteria: • LSU: > 97% identity; MycoBank (16/20) • ITS: > 99% identity; NCBI nt (14/20)

Note: The validation was performed either using a collection of DNA extracts stored in a freezer or using mould cultures from which DNA had been recently extracted.

APPENDIX 1 – REFERENCE MANUALS USED FOR MOULD IDENTIFICATION

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