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**ABSTRACT**

Food poisoning caused by toxic mushrooms, such as species in the *Amanita* genus, occurs frequently around the world. To properly treat these patients, it is important to rapidly and accurately identify the causal species. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry is a rapid technique that has been used in medical laboratories for the past three decades to identify bacteria, yeasts and filamentous fungi.

Matrix Assisted Laser Desorption Ionisation Time of flight mass spectrometry (MALDI-Tof MS) is a rapid method used for the past three decades to identify microorganisms. In this study, we created and internally validated a MALDI-Tof MS reference database comprising 15 *Amanita* species frequently encountered in France, and we challenged this database with 38 *Amanita* specimens from four French locations, using a free online application for MALDI-ToF spectra identifications.

Assessment of the database showed that mass spectra can be obtained by analyzing any portion of a carpophore and that all portions enabled identification of the carpophore at the species level. Most carpophores were correctly identified using our database, with the exception of specimens from the *Vaginatae section*. Decay tests also demonstrated that decayed portions (like those found in the kitchen garbage can) of *Amanita phalloides* mushrooms could be properly identified using MALDI-ToF MS.

Our findings provide important insight for toxicology laboratories that often rely on DNA sequencing to identify meal leftovers implicated in food poisoning. In future developments, this technique could also be used to detect counterfeit mushrooms by including other genera in the reference database.

## LAY SUMMARY

MALDI-ToF MS is a powerful identification tool for microorganisms. We demonstrate that the technique can be applied to Amanita specimens. This will prevent food intoxications as a rapid and definite identification can be obtained, and it can also be used for food remnants.

## INTRODUCTION

Mushrooms have been implicated in many cases of food poisoning globally. Most life-threatening poisoning cases due to ingestion of toxic mushrooms are caused by specimens belonging to the *Amanita* genus, especially *A. phalloides, A. virosa, A. verna* and *A. decipiens. A. phalloides* is responsible for approximately 95% of poisoning cases globally [1]. Various symptoms may develop depending on the species of mushroom ingested [2,3,4(p)]. Most symptoms induced by *Amanita* mushrooms are caused by amatoxins that block DNA production, thereby leading to cell death, especially in cells that require frequent renewal (e.g., liver and kidney cells). Other mycotoxins have also been implicated in cases of mushroom poisoning, such as coprine, muscarine, muscimol, ibotenic acid and gyromitrin [2]. The toxins in certain mushrooms can cause Phalloidic syndrome, in which the initial symptoms may include respiratory difficulties and vertigo, followed by painful vomiting, acute diarrhea and eventual severe dehydration. Subsequent symptoms may include severe toxic hepatitis, which can lead to liver destruction, followed by cerebral disorders. Patients may recover after several months of convalescence; however, up to approximately 30% of cases succumb to the intoxication [4–14].

Identification of species implicated in food poisoning is important to properly treat patients, as the symptoms and appropriate treatment vary depending on the species involved [2].

Mushroom identification is a complex process that requires specialists, as the toxic potential of morphologically similar mushrooms is difficult to assess (e.g., *Amanita spissa* and *A. rubescens*

mushrooms are non-toxic and edible if thoroughly cooked, while phenotypically look alike *A. pantherina* carpophores can be deadly). When analyzing meal leftovers, species identification based on morphology is nearly impossible and thus requires DNA sequence analysis. However, this technique remains relatively expensive and time-consuming, requiring one to several days to obtain results, especially if food samples contain several species. Real-time PCR assays have been developed to detect the presence of some *Amanita* species in food remnants or human feces, although the method is only specific for three species (i.e., *A. phalloides*, *A. virosa* and *A. verna*) [15]. Consequently, toxicologists recommend treating the symptoms in food poisoning cases before attempting to identify the species, as these identification methods are often either uninformative or too time consuming [16].

The MALDI-ToF mass spectrometry technique has been applied in the mycology domain to efficiently identify fungal species such as yeasts, molds and dermatophytes [17–24]. However, to the best of our knowledge, this method has only been used once to identify mushroom species, involving a diverse panel of mushrooms from Hokkaido Island in Japan [25]. An online identification application (MSI) has recently been created to construct reference databases of various organisms and make them available for laboratories worldwide [26,27]. This application allows users from around the world to freely and rapidly identify spectra obtained from yeasts, filamentous fungi, dermatophytes, parasites (such as Leishmania), or insects (such as Phlebotomes) since 2017. More than 1000 references of various yeasts, filamentous fungi, sand fly and parasite species are already available in the application.

In this study, we developed a database to easily and rapidly identify mushrooms of the *Amanita* genus using MALDI-ToF mass spectrometry and the MSI online application. We present here a proof of concept that highlights the possibility to easily and rapidly identify mushrooms from a

very small amount of mushroom material, using a combination of Maldi-ToF mass spectrometry technology and the online application (MSI).

## METHODS

**Construction of the MSI reference database**.

**Carpophore collection**. The local mycologist association *Société Mycologique de Provence* (SMP) in Marseille Provence (southeastern France) collected mushrooms to be included in the reference database. From September 2013 to October 2016, a total of 447 carpophores were collected in the Provence region by members of the SMP. In 2015, 3 *Amanita* carpophores were collected in the Vosges Forest (located in northeastern France). In October 2016, the local mycologist association (Cercle d'études mycologiques en Aquitaine) in Bordeaux (southwestern France) collected 34 carpophores. In 2017, 3 *Amanita* carpophores were collected in the Doubs Forest (northeastern France). In total, 487 carpophores were collected for the study.

**Carpophore identification**. The local mycologist associations conducted phenotype-based identification of the carpophores. Accordingly, assessment of all 484 carpophores revealed 128 genera and 366 species.

Each carpophore was also submitted for DNA sequence analysis of the ITS1-ITS2 region using the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers, according to the protocol described by Fujita et al [28], with an annealing temperature of 55°C. Identification at the species level was confirmed using a phylogenetic tree and the reference identifications published by Cui et al. in 2020 [29] (supplemental figure 1).

**Construction of the mass spectrometry reference database**. The *Amanita* reference database comprised spectra obtained using a Microflex LT system (Bruker Daltonics, Bremen, Germany). To analyze each *Amanita* carpophore, a 1 x 2-mm sample was extracted from 2 to 3 gills. A small sample of the stem, ring and volva portions (approximately 2 $mm^3$) were also extracted in

separate tubes when available and relevant. The mushroom samples were suspended in 75% ethanol HPLC to inactivate the mushroom. After a 10-minute centrifugation step at 13,000 g, the hydroalcoholic solution was removed, and the pellets were suspended in at least 20 µl of 70% formic acid (or enough volume to cover the pellet) (Sigma-Aldrich, France). The mushroom samples were homogenized in formic acid by compressing the sample against the wall of the tube and pipetting up and down. After a 5-minute incubation step that allows the cell walls to be destroyed in contact with the formic acid, the same volume of acetonitrile HPLC was added (VWR International S.A.S., Fontenay-sous-Bois, France), and the two reagents were mixed by pipetting up and down. After 5 minutes of incubation at ambient temperature for neutralization of the acid and precipitation of the proteins, the sample was centrifuged for 2 minutes at 13,000 g, and 1 µl-drops of the supernatant were deposited onto the polished steel targets (MTP384, Bruker Daltonics GmbH, Bremen, Germany). Each deposit was then covered with 1 µl of matrix (α-cyano-4-hydroxycinnamic acid (HCCA)) (Applied BiosystemsW, Villebon sur Yvette, France). Four to 10 deposits were realized for each portion of each carpophore that was sampled, and the spectra were acquired using the original Bruker parameters. To compensate for the difficulties encountered when acquiring stem spectra, previous studies have proposed to modify either the software parameters or the extraction protocol [25,30]. We chose to modify the parameter in the Flexcontrol software for all mushroom samples, in which the "save sum of rejected spectra" parameter was selected instead of "do not save", or "save zeroline" in the "behavior after unsuccessful acquired spectrum" parameter (as previously suggested in a study on filamentous fungi [30]). Each acceptable spectrum (i.e., visible separated peaks) was considered as a reference spectrum, and a reference database was generated in the updated version of the MSI application (https://msi.happy-dev.fr/) [27].

**Score determination**

Each spectrum that was retained as a reference was compared against all other references using the MSI application. The comparison scores were assigned a 'validity label' as follows: "concordant at the species level" (reference of the same species), "concordant at the series level" (reference belonging to the same series but not the same species) or "discordant" (reference belonging to a different series). The best identification used for score determination was the first one belonging to another carpophore than the targeted spectrum.

**Identification threshold**

The identification threshold was determined using two graphs (figure 1 and figure 2). The first graph (figure 1) compiled, for each spectrum, three data: i/the best scores that were concordant at the species level, ii/concordant at the series level and iii/discordant. The second graph (figure 2) categorized only the best score for each spectrum according to the validity label. The threshold defined for the study was the minimum score for which there was no error of identification at the species level. After determining the threshold, any result with a score below the threshold was not considered for analysis and was labeled 'no identification'.

**Identification scores per portion**

As each of the spectra used to generate the reference database was used to challenge the database, we collected, among the correct identifications at the species level, the best scores obtained against the various available portions of the same species. Thus, for a stem spectrum, we could obtain up to four scores, one against the stem portion (if not belonging to the same carpophore), one against the gills portion, one against the volva portion and one against the ring portion (from any carpophore). For all spectra, the mean scores of each portion were calculated to evaluate their identification potential.

**Performance tests**

1/ Using the MSI application, we compared the database against a validation sample of 9 carpophores (stem portions only) belonging to the same mycelium (i.e., collected at the same time and in the same location, belonging to the same fairy ring) as the carpophores used for the reference database. The validation sample corresponded to 6 *Amanita* species: *A. citrina*, *A. muscaria*, *A. ovoidea*, *A. pantherina*, *A. phalloides* and *A. rubescens*.

2/ Using the MSI application, we also compared the database against an external test sample of 44 carpophores (73 portions) collected during a six-year period from four regions in France (32 samples from the Vosges Forest between 2013 and 2019, 1 sample from Provence in 2016, 8 samples from the Paris area in 2019 and 3 samples from the Doubs Forest in 2017).

These carpophores were initially identified either via phenotyping or DNA sequencing. Seven *Amanita* taxa were used for these external tests (*A. citrina* (n=15), *A. rubescens* (n=11), *A. muscaria* (n=8), *A. af. Vaginatae* section (n=7), *A. pantherina* (n=1), *A. submaculata* (n=1) and *A. junquillea* (n=1)). If the best score obtained was below the threshold defined in the first part of this study, the spectrum was considered as not identified. If the score was above the threshold, the identification result was compared to the phylogenetic identification obtained from the ITS DNA sequencing, to determine whether it was correct or not.

**Decay test.** A decay test was performed on 2 *A. phalloides* carpophores for several days until the sample decayed to a liquefied state at room temperature. Spectra from each portion were compared from one day to the next using the Bruker Flex Analysis software. We then generated a small-scale decay reference database with these spectra for analysis using the MSI application.

## RESULTS

**References.** After assessing the DNA sequences and comparing the DNA results against the phenotype results, we identified a total of 41 carpophores that corresponded to the *Amanita* genus and 14 *Amanita* species. These carpophores belonged to 8 series or species subsections as

follows: the *Amanita* series (*A. muscaria*), the *Pantherina* series (*A. pantherina,* and *A. junquillea*), the *Caesareae* subsection (*A. caesarea*), the *Vaginatae* section (*A. submembranacea*), the *Validae* series (*A. rubescens* and *A. spissa*), the *Mappae* series (*A. citrina*, *A. intermedia* and *A. porphyria*), the *Ovoidea* series (*A. ovoidea* and *A. proxima*) and the *Phalloideinae* subsection (*A. phalloides* and *A. verna-var-decipiens*). Each species was represented by 1 to 6 carpophores, and most references were obtained from specimens collected in Provence (31/41). A total of 102 *Amanita* portions were used to generate 815 individual reference spectra. The details for the references of each of the 14 *Amanita* species are provided in Table 1.

**Identification threshold.**

We found that threshold scores above 18.64 consistently corresponded to correct identification results (with the exception of an *A. submembranacea* stem spectrum that initially matched an *A. porphyria* reference with a score of 25.05 (Figures 1 and 2).

Applying a score threshold of 20, 74% of the tested spectra were correctly identified, of which all but one were correct at the species level.

**Identification scores per portion.**

The identification scores were higher when comparing the same portion types (i.e., stem *vs.* stem) than when comparing different portion types (i.e., stem *vs.* gills) (Table 2). Nevertheless, all portion references enabled identification of spectra from the other portions, with the exception of the ring reference for which the identification threshold was not reached for the volva spectra and vice versa. As those two portions could be efficiently identified by the stem and gills references, we determined that stem and gill spectra are better suited to construct an efficient reference database.

**Identification results.**

Identification of carpophores from the same mycelium.

We analyzed 9 stems from carpophores that corresponded to the same mycelium of at least one reference in the database. Four spectra were acquired for each stem and were all correctly identified with identification scores ranging from 27.25 (*A. muscaria*) to 58.13 (*A. citrina*) (Table 3). Only 3 of 36 spectra did not reach the identification threshold and thus could not be identified.

Identification of carpophores from different mycelia.

The results obtained for the 44 carpophores (73 portions) from mycelia unrelated to other mycelia in the database are detailed in Table 4.

Among these 44 carpophores, the 7 specimens belonging to the *Vaginatae* section (5 *A. fulva,* 1 *A. umbrinolutea* and 1 unidentified) were not identified, as only *A. submembranacea* represents this section in the references database. Two of the remaining 37 carpophores were either not identified (score <20) or misidentified: 1 *A. submaculata* sample did not reach the defined threshold (this species was not represented in the database), and 1 *A. rubescens* sample from the Vosges Forest in 2019 yielded scores below the defined threshold (19.3 for the gills and 18.24 for the stem).

The other 35 carpophores were correctly identified by analyzing at least one portion. These 35 carpophores were represented by 57 portions, among which 52 (91%) were identified with a score above the defined threshold. Identification difficulties were observed with the carpophores collected in August 2019, which had very dry stems and uncharacteristic morphology due to the drought that occurred that year.

**Decay test.**

Two *A. phalloides* carpophores were tested over the course of three consecutive days until the samples decayed. Spectra were obtained from the stem, gill and volva portions.

233 On decay day 3, the spectra appeared to show fewer peaks than on day 1 or day 2 (Figure 3).
234 Assessment of the stem profiles revealed that most peaks visible on day 1 seemed to remain over
235 the next two days, albeit with reduced intensity. The gill and volva profiles seemed to have fewer
236 peaks than the stem profiles between decay days 2 and 3. When assessing these spectra using the
237 MSI application (Figure 4), we found that all references enabled identification of all decay
238 sample spectra. However, the references obtained on day 2 enabled the most efficient
239 identification of the spectra from decay days 1 to 3.

240 **DISCUSSION**

241 Identification of toxic mushrooms at the species level is important to better diagnose and treat
242 cases of food poisoning due to toxic and potentially deadly mushroom species [4,31]. Phenotype-
243 based identification of mushroom species should be performed by a specialist with expertise in
244 fungal taxonomy and morphology. Currently, DNA analysis techniques are the only widely
245 accepted alternative to phenotype-based identification, either by sequencing various portions of
246 specific genes or using real-time PCR assays designed to recognize a few species of interest [15].
247 The primary objective of this study was to assess whether MALDI-ToF mass spectrometry, a
248 rapid and less expensive technique than DNA-based methods, could be applied to identify
249 mushrooms at the species level. We focused on the *Amanita* genus, which has been implicated in
250 most life-threatening mushroom poisoning cases reported in France [4,8,32].
251 We successfully obtained spectra from all portions of the carpophores using MALDI-ToF mass
252 spectrometry. To circumvent the difficulties encountered when acquiring stem spectra, we found
253 that modification of an acquisition parameter in the Flex Control software was sufficient and that
254 modification of the extraction protocol was unnecessary. Assessment of the database enabled us
255 to define an identification threshold of 20, which is similar to that previously defined for
256 pathogenic filamentous fungi [27]. While testing the database by comparing the reference spectra

against the same spectra in a test panel, we often found that spectra obtained from different portions of the same carpophore varied but showed sufficient similarity to enable correct identification at the species level. Comparison between the ring and volva spectra revealed the lowest degree of similarity.

After defining the identification threshold, we submitted 36 spectra of carpophores belonging to the same mycelia as references for the MSI application. When considering an identification threshold of 20, identification performance was excellent with scores reaching a maximum of 76.3. When considering only the best identification results of the four spectra submitted for each mushroom sample, 100% of the samples were correctly identified.

External validation of the database was performed using carpophores collected in regions other than those represented in the reference database. The validation revealed that 80% (35/44) of the carpophores were identified by assessing at least one portion, and 71% (52/73) of the portion samples were correctly identified.

The majority of misidentifications (7 carpophores) were associated with the section *Vaginatae*, which comprises a large number of species that were not included in our reference database, and which is continuously evolving. In 2009, Neville and Poumarat [33] described over 50 species in this subsection, while new species have been described since then by other authors [29,34,35]. In 2020, Hanss and Moreau compiled a total of 86 species in the *Vaginatae* section, using ITS DNA sequencing [36], and there are currently 96 provisional names recorded by Tulloss and Yang [37] in this section. Species-level identification of carpophores from the *Vaginatae* section using DNA databases can also be challenging, as new species have recently been phenotypically described and DNA sequences are not always available. However, when this subsection was excluded, 95% (35/37) of the carpophores and 85% (52/61) of the portions were correctly identified.

The samples collected in 2019 presented another challenge due to the drought that occurred that summer. As the stems of these mushrooms were drier than usual, many of the 2019 samples did not reach the identification threshold of 20. When only considering species represented in the database (i.e., not including *the Vaginatae section*), the identification rate was 87.5% for samples from 2013 (14/16 portions), 100% for samples from 2017 (9/9 portions) and 82% for samples from 2019 (24/29 portions). These results suggest that identification performance could be improved by including specimens collected during droughts or in particular conditions (e.g., altitude, humidity, pollution) in the database.

The decay test performed on the *A. phalloides* specimens showed that the spectra could be identified regardless of the advanced state of decay. As the references obtained on a specific day enabled identification of spectra obtained from the day before and the day after, it is unnecessary to generate a database of decayed sample references obtained each day. As the timeframe of symptom development varies depending on the species implicated, mushroom databases should consider at least one reference of each portion every two days during the decay phase. It is important that the protein profile of the mushroom portion most likely to be recovered from the garbage does not markedly evolve in the decay process. MALDI-ToF mass spectrometry should help toxicologist to identify the cause of the poisoning regardless of the stage of decay [16].

In conclusion, this study shows that mushrooms, and in this case the *Amanita* genus, can be rapidly and consistently identified at the species level using MALDI-ToF mass spectrometry and the MSI application, even on decayed samples. This study provides important insight for toxicology laboratories that often utilize DNA sequencing to identify pathogenic mushrooms and diagnose patients. In future developments, such MALDI-ToF-based identification approaches could also be used to detect counterfeit mushrooms.

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**Conflicts of Interest : None**

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## FIGURES LEGENDS

**Figure 1**: Best concordant at the species level, concordant at the series level and discordant scores for each spectrum.

**Figure 2**: Repartition of the best score for each spectrum.

**Figure 3**: Evolution of the decay spectra for each portion for the two *A. phalloides* carpophores.

**Figure 4**: Score distribution for the various decay days (white boxes: spectra realized at decay day 1; gray boxes: spectra realized at decay day 2; black boxes: spectra realized at decay day 3). The lower and upper portions of the box represent the lower and upper quartiles, respectively. The line represents the median value. The ends of the whiskers represent the lowest datum included in the 1.5 interquartile range of the lower quartile and the highest datum included in the 1.5 interquartile range of the upper quartile. Outlier values are represented by circles. For each spectrum, scores noted correspond to the identification against the same specimen but at different decay days. Therefore, for each spectrum, three scores were used: decay day 1, decay day 2 and decay day 3.

**Supplemental Figure 1**: Phylogeny using ITS sequences of the specimens collected in this study, using the maximum likelihood (ML) analysis, and 1000 bootstraps. Bootstrap values are represented by stars, and values below 70 are not shown. Sequences from Cui et al. [29] are highlighted in red and displayed in italic. Specimens used as MS references are displayed in bold.

455 **Table 1**: Details of the references obtained for each of the 15 *Amanita* species

| Species | Carpophore origin | NCBI accession number for ITS sequences | Number of portions / number of stem references | Number of portions / number of gill references | Number of portions / number of volva references | Number of portions / number of ring references | Number of portions / number of total references | Harvest year | Decay test |
|---|---|---|---|---|---|---|---|---|---|
| *A. caesarea* | Provence (1) | MW589070 | 1 / 10 | 1 / 10 | 1 / 10 | 1 / 10 | 4 / 40 | 2013 (1) | No |
| *A. citrina* | Provence (6)<br><br><br><br><br>Vosges (1) | MW589071*<br>MW589072<br>MW589073<br>MW589074<br>MW589075 | 7 / 52 | 7 / 52 | 0 / 0 | 2 / 20 | 16 / 124 | 2013 (2),<br>2014 (1),<br>2015 (1),<br>2016 (3) | No |
| *A. intermedia* | Provence (1) | MW589076 | 1 / 10 | 1 / 10 | 0 / 0 | 0 / 0 | 2 / 20 | 2013 (1) | No |
| *A. junquillea* | Provence (3) | MW589077<br>MW589078* | 3 / 18 | 3/18 | 0 / 0 | 0 / 0 | 6 / 36 | 2014 (1),<br>2016 (2) | No |
| *A. muscaria* | Provence (3)<br><br><br>Bordeaux (1)<br><br>Vosges (1) | MW589079<br>MW589080<br>MW589081<br>No sequence available<br>MW589082 | 4 / 34 | 5 / 44 | 1 / 4 | 1 / 10 | 11 / 92 | 2013 (2),<br>2014 (1),<br>2015 (1),<br>2016 (1) | No |
| *A. ovoidea* | Provence (2) | No sequence available | 2 / 19 | 2 / 20 | 0 / 0 | 0 / 0 | 4 / 39 | 2013 (2) | No |
| *A. pantherina* | Provence (1)<br>Bordeaux (2) | MW589083<br>MW589126* | 3 / 18 | 3 / 18 | 0 / 0 | 0 / 0 | 6 / 36 | 2013 (1),<br>2016 (2) | No |
| *A. phalloides* | Provence (4)<br><br><br><br>Bordeaux (2) | MW589084<br>MW589085<br>MW589086<br>MW589087<br>MW589127* | 10 / 88 | 10 / 88 | 6 / 60 | 0 / 0 | 26 / 236 | 2013 (3),<br>2014 (1),<br>2016 (2) | Day 1, day 2 and day 3 |
| *A. porphyria* | Provence (3) | MW589088* | 3 / 12 | 3 / 12 | 0 / 0 | 0 / 0 | 6 / 24 | 2016 (3) | No |
| *A. proxima* | Provence (1) | MW589089 | 1 / 10 | 1 / 10 | 0 / 0 | 0 / 0 | 2 / 20 | 2013 (1) | No |
| *A. rubescens* | Provence (2)<br><br>Bordeaux (2) | MW589091<br>MW589092<br>MW589128* | 4 / 28 | 4 / 28 | 1 / 4 | 0 / 0 | 9 / 60 | 2014 (2),<br>2016 (2) | No |
| *A.spissa* | Provence (1) | MW589090 | 1/10 | 1/10 | 0/0 | 0/0 | 2/20 | 2013 (1) | No |
| *A. submembranacea* | Provence (1)<br>Vosges (1) | MW589094<br>MW589093 | 2 / 14 | 2 / 14 | 0 / 0 | 0 / 0 | 4 / 28 | 2015 (1),<br>2016 (1) | No |
| *A. verna var decipiens* | Provence (2) | MW589095 | 2 / 20 | 2 / 20 | 0 / 0 | 0 / 0 | 4 / 40 | 2016 (2) | No |

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457 *several different carpophores from the same mycelium incorporated as references, only one

458 sequenced.

459 **Table 2**: Mean and standard deviation of the scores obtained for each spectrum against a
460 reference of each portion of the same species. The number of spectra that yielded a correct
461 identification against the specified portion is indicated in parentheses.

462

| Reference portion / Portion spectra | Ring mean score +-st dev | Stem mean score +-st dev | Gill mean score +-st dev | Volva mean score +-st dev |
|---|---|---|---|---|
| Ring (n=40) | 78.13 ± 11.61 (n=40) | 29.77 ± 6.45 (n=40) | 29.23 ± 6.22 (n=39) | 16.54 ± 4.11 (n=20) |
| Stem (n=343) | 21.96 ± 8.73 (n=94) | 76.15 ± 13.39 (n=323) | 29.95 ± 9.85 (n=302) | 27.83 ± 12.10 (n=136) |
| Gill (n=354) | 23.63 ± 7.69 (n=103) | 29.07 ± 9.60 (n=301) | 70.11 ± 16.04 (n=334) | 22.51 ± 9.81 (n=150) |
| Volva (n=78) | 13.51 ± 5.78 (n=14) | 34.31 ± 9.99 (n=74) | 27.33 ± 8.61 (n=73) | 67.69 ± 15.36 (n=78) |

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466 **Table 3**: Results for nine carpophores belonging to the same mycelium of at least one other

467 reference in the database.

| Carpophore sample | Portion | Identified species | Maximum score | Validation |
|---|---|---|---|---|
| Provence2013-M124_*A. muscaria* | stem | *Amanita muscaria* | 55.26 | Correct |
| Provence2013-M127_*A. citrina* | stem | *Amanita citrina* | 76.3 | Correct |
| Provence2013-M132_*A. citrina* | stem | *Amanita citrina* | 65.53 | Correct |
| Provence2013-M004_*A. intermedia* | stem | *Amanita intermedia* | 42.63 | Correct |
| Provence2013-M015_*A. muscaria* | stem | *Amanita muscaria* | 29.28 | Correct |
| Provence2013-M166_*A. pantherina* | stem | *Amanita pantherina* | 54.42 | Correct |
| Provence2013-M171_*A. rubescens* | stem | *Amanita rubescens* | 53.3 | Correct |
| Provence2013-M174_*A. ovoidea* | stem | *Amanita ovoidea* | 29.3 | Correct |
| Provence2013-M180_*A. phalloides* | stem | *Amanita phalloides* | 47.15 | Correct |

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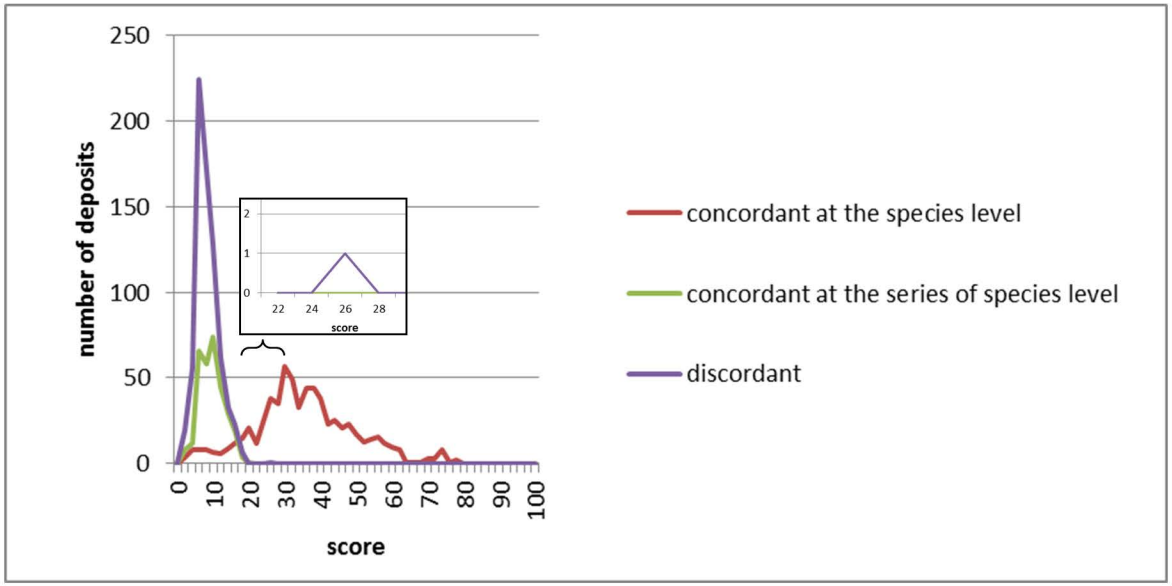
**Table 4**: Results for the 44 carpophores from mycelia unrelated to other mycelia in the database. Identification results with scores below 20 were not considered due to the high likelihood of misidentification.

| Sample-DNA identification | NCBI accession number for ITS sequences | Portion | MSI identification | Maximum score | Validation |
|---|---|---|---|---|---|
| Doubs2017-A_*A. citrina* | No sequence available | stem | *Amanita citrina* | 33.9 | Correct |
| Doubs2017-B_*A. muscaria* | No sequence available | stem | *Amanita muscaria* | 28.6 | Correct |
| Doubs2017-C_*A. rubescens* | No sequence available | stem | *Amanita rubescens* | 25.3 | Correct |
| Paris2019-A_*A. citrina* | MW589096 | ring | *Amanita citrina* | 21.1 | Correct |
| | | stem | *Amanita citrina* | 23.2 | Correct |
| | | gill | *Amanita citrina* | 30.4 | Correct |
| | | volva | Unidentified | 0 | No identification |
| Paris2019-B_*A. af. Vaginatae section* | No ITS sequence available | stem | Unidentified | 0 | No identification |
| | | gill | Unidentified | 0 | No identification |
| Paris2019-C_*A. muscaria* | MW589097 | stem | *Amanita muscaria* | 48.2 | Correct |
| | | gill | *Amanita muscaria* | 52.1 | Correct |
| Paris2019-D_*A. pantherina* | No ITS sequence available | stem | Unidentified | 0 | No identification |
| | | gill | *Amanita pantherina* | 33.3 | Correct |
| Paris2019-E_A. *A. rubescens* | No ITS sequence available | ring | *Amanita rubescens* | 31.4 | Correct |
| | | stem | *Amanita rubescens* | 30.1 | Correct |
| | | gill | *Amanita rubescens* | 37.2 | Correct |
| Paris2019-F_*A. submaculata* | MW589098 | stem | Unidentified | 0 | No identification |
| | | gill | Unidentified | 0 | No identification |
| Paris2019-G_*A. rubescens* | MW589099 | stem | *Amanita rubescens* | 32.3 | Correct |
| | | gill | *Amanita rubescens* | 32.4 | Correct |
| Paris2019-H_*A. citrina* | No sequence available | stem | *Amanita citrina* | 36.8 | Correct |
| | | gill | *Amanita citrina* | 49.1 | Correct |
| Provence2016-M338_*A. muscaria* | MW589100 | stem | *Amanita muscaria* | 46.7 | Correct |
| | | gill | *Amanita muscaria* | 46.4 | Correct |
| Vosges2013-C_*A. rubescens* | MW589101 | stem | Unidentified | 26.9 | Correct |
| | | gill | *Amanita rubescens* | 31.5 | Correct |
| Vosges2013-D_*A. fulva* | MW589102 | stem | Unidentified | 0 | No identification |
| | | gill | Unidentified | 0 | No identification |
| Vosges2013-E_*A. muscaria* | MW589103 | stem | *Amanita muscaria* | 52.9 | Correct |
| | | gill | *Amanita muscaria* | 43.7 | Correct |
| Vosges2013-F_*A. citrina* | MW589104* | stem | *Amanita citrina* | 40.7 | Correct |
| | | gill | *Amanita citrina* | 46.5 | Correct |
| Vosges2013-F1_*A. citrina* | | gill | *Amanita citrina* | 58.4 | Correct |
| Vosges2013-F2_*A. citrina* | | gill | *Amanita citrina* | 43.6 | Correct |
| Vosges2013-G_*A. citrina* | MW589105* | stem | *Amanita citrina* | 23.1 | Correct |
| | | gill | *Amanita citrina* | 457 | Correct |
| Vosges2013-G1_*A. citrina* | | gill | *Amanita citrina* | 30.5 | Correct |
| Vosges2013-G2_*A. citrina* | | gill | Unidentified | 29.3 | Correct |
| Vosges2013-H1_*A. junquillea* | MW589106 | stem | Unidentified | 0 | No identification |
| | | gill | *Amanita junquillea* | 21.6 | Correct |
| Vosges2013-H2_*A. citrina* | MW589107 | stem | Unidentified | 36.1 | Correct |
| | | gill | *Amanita citrina* | 19 | No identification |
| Vosges2017-A_*A. citrina* | MW589108 | gill | *Amanita citrina* | 49.3 | Correct |
| Vosges2017-B_*A. citrina* | MW589109 | gill | *Amanita citrina* | 46.6 | Correct |
| Vosges2017-C_*A. citrina* | MW589110 | gill | *Amanita citrina* | 42 | Correct |
| Vosges2017-D_*A. muscaria* | MW589111 | gill | *Amanita muscaria* | 46.7 | Correct |
| Vosges2017-E_*A. muscaria* | MW589112 | gill | *Amanita muscaria* | 55 | Correct |
| Vosges2017-F_*A. muscaria* | MW589113 | gill | *Amanita muscaria* | 48.1 | Correct |
| Vosges2017-G_*A. fulva* | MW589114 | gill | Unidentified | 0 | No identification |

| | | | | | |
|---|---|---|---|---|---|
| Vosges2017-H_*A. umbrinolutea* | MW589115 | gill | Unidentified | 0 | No identification |
| Vosges2017-I_*A. rubescens* | No sequence available | gill | *Amanita rubescens* | 36.9 | Correct |
| Vosges2017-J_*A. rubescens* | No sequence available | gill | *Amanita rubescens* | 39.8 | Correct |
| Vosges2017-K_*A. rubescens* | No sequence available | gill | *Amanita rubescens* | 28.5 | Correct |
| Vosges2019-A_*A. fulva* | MW589116 | stem | Unidentified | 0 | No identification |
| | | gill | Unidentified | 0 | No identification |
| Vosges2019-B_*A. rubescens* | MW589117 | stem | *Amanita rubescens* | 18.3 | No identification |
| | | gill | *Amanita rubescens* | 41.3 | Correct |
| Vosges2019-C_*A. fulva* | MW589118 | stem | Unidentified | 0 | No identification |
| | | gill | Unidentified | 0 | No identification |
| Vosges2019-D_*A. rubescens* | MW589119 | stem | Unidentified | 17.1 | No identification |
| | | gill | *Amanita rubescens* | 19.3 | No identification |
| Vosges2019-E_*A. citrina* | MW589120 | stem | *Amanita citrina* | 31.7 | Correct |
| | | gill | *Amanita citrina* | 39.2 | Correct |
| Vosges2019-F_*A. citrina* | MW589121 | stem | *Amanita citrina* | 45 | Correct |
| | | gill | *Amanita citrina* | 49.3 | Correct |
| Vosges2019-G_*A. fulva* | MW589122 | stem | Unidentified | 0 | No identification |
| | | gill | Unidentified | 0 | No identification |
| Vosges2019-H_*A. muscaria* | MW589123 | stem | *Amanita muscaria* | 47.9 | Correct |
| | | gill | *Amanita muscaria* | 44 | Correct |
| Vosges2019-I_*A. rubescens* | MW589124 | stem | *Amanita rubescens* | 24.8 | Correct |
| | | gill | *Amanita rubescens* | 40.3 | Correct |
| Vosges2019-J_*A. rubescens* | MW589125 | stem | *Amanita rubescens* | 35.7 | Correct |
| | | gill | *Amanita rubescens* | 34.7 | Correct |

*several different carpophores from the same mycelium incorporated as references, only one sequenced.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150
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number of deposits
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10
20
30
40
50
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70
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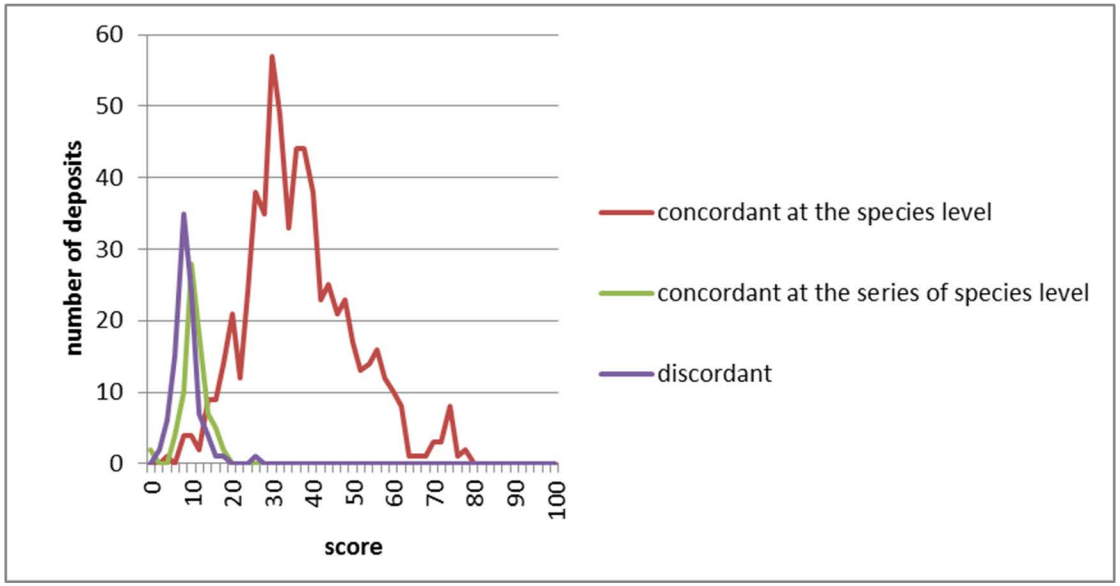
1
0
22
24
26
28
score
concordant at the species level
concordant at the series of species level
discordant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30
40
50
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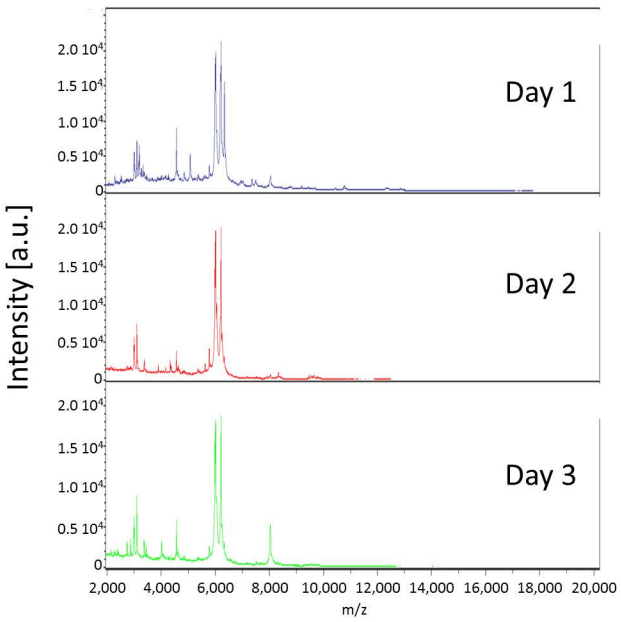
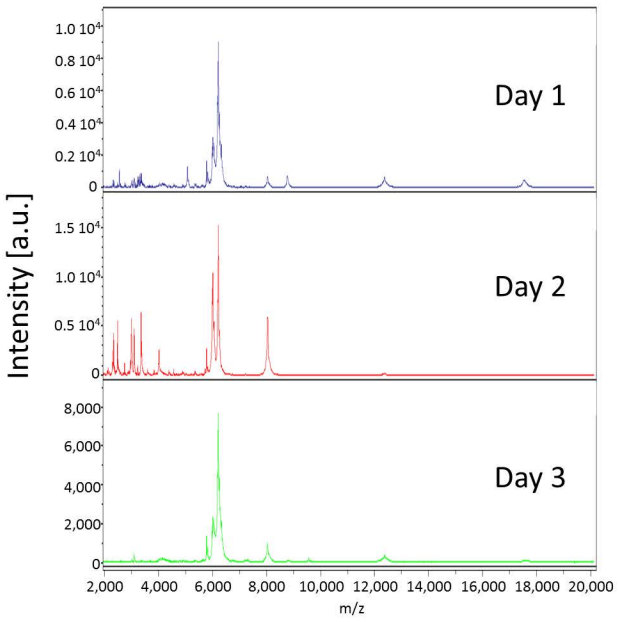
80
90
100
score
concordant at the species level
concordant at the series of species level
discordant

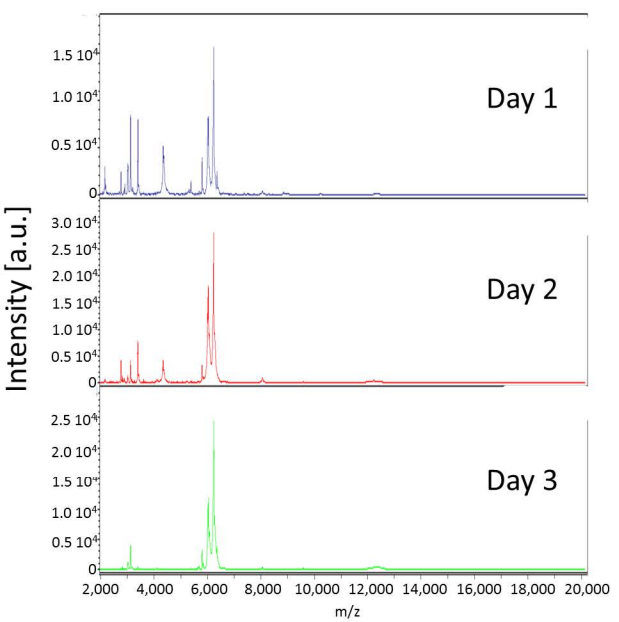
*Amanita phalloides* M150 Stem portion



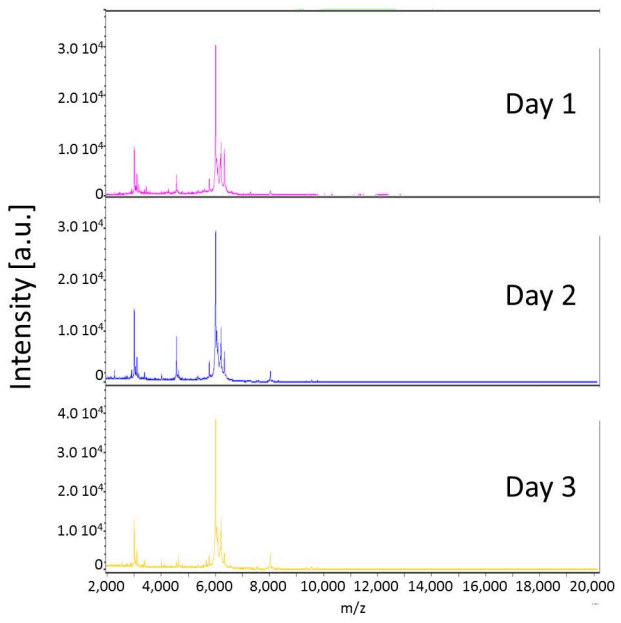

*Amanita phalloides* M150 Gills portion



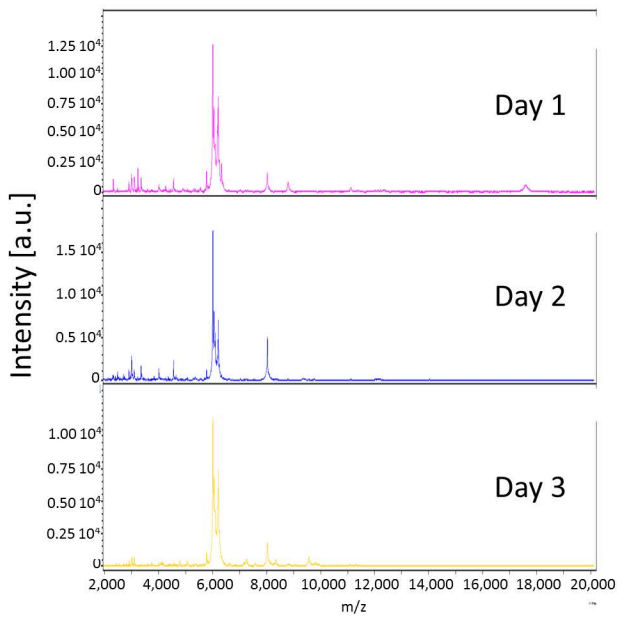

*Amanita phalloides* M150 Volva portion



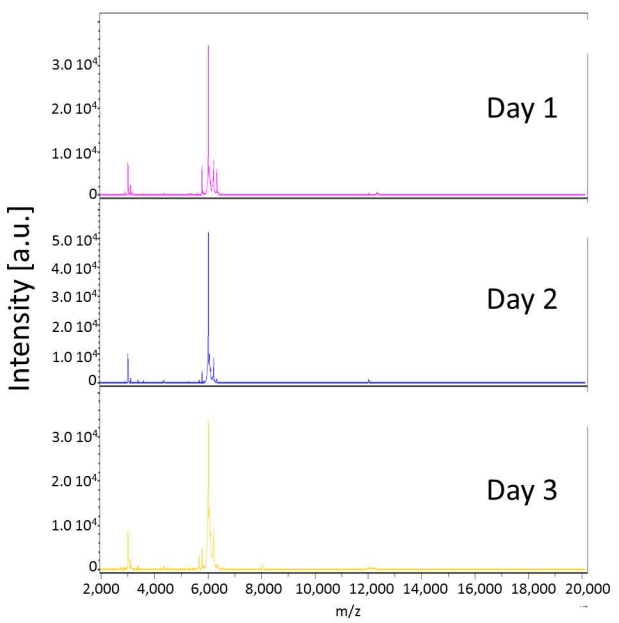

*Amanita phalloides* M160 Stem portion

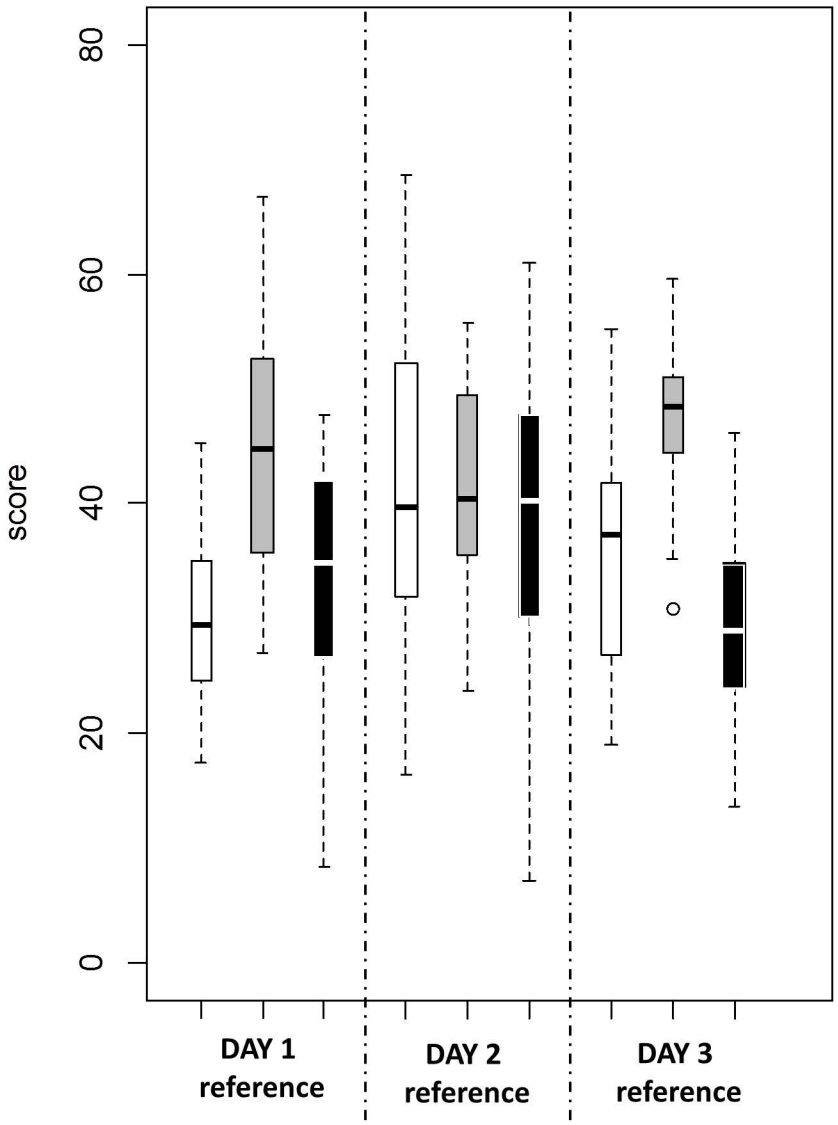



*Amanita phalloides* M160 Gills portion




*Amanita phalloides* M160 Volva portion

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60
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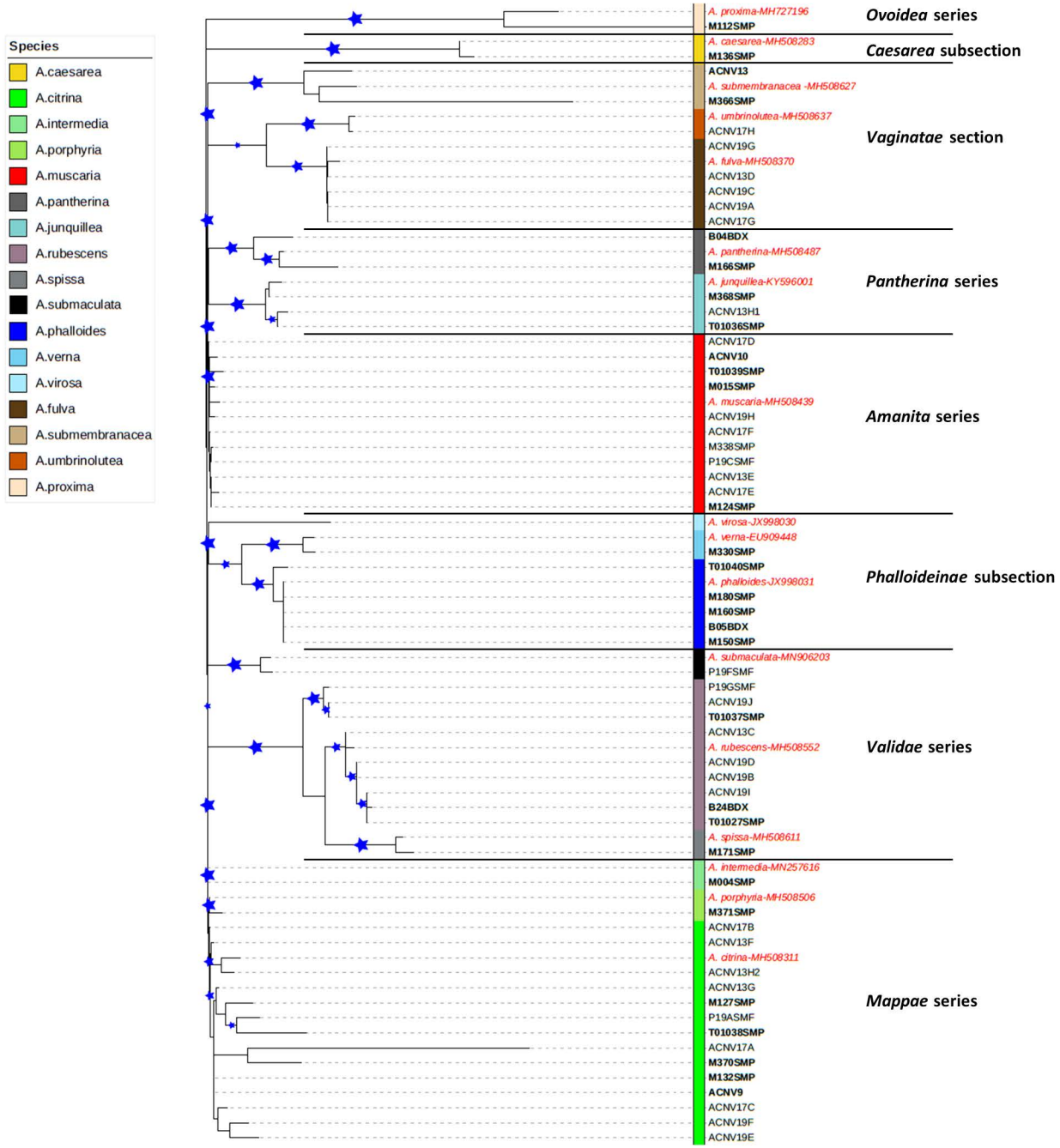
reference
DAY 2
reference
DAY 3
reference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A.spissa
A.submaculata
A.phalloides
A.verna
A.virosa
A.fulva
A.submembranacea
A.umbrinolutea
A.proxima
A. proxima-MH727196
M112SMP
A. caesarea-MH508283
M136SMP
ACNV13
A. submembranacea -MH508627
M366SMP
A. umbrinolutea-MH508637
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ACNV19G
A. fulva-MH508370
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ACNV19C
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B04BDX
A. pantherina-MH508487
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A. porphyria-MH508506
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ACNV17B
ACNV13F
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M370SMP
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ACNV9
ACNV17C
ACNV19F
ACNV19E
Ovoidea series
Caesarea subsection
Vaginatae section
Pantherina series
Amanita series
Phalloideinae subsection
Validae series
Mappae series