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1 **Using MALDI-ToF mass spectrometry to identify mushroom species – Proof of concept**  
2 **analysis of *Amanita* genus specimens**

3  
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21 **ABSTRACT**

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

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

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

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

43

44 **LAY SUMMARY**

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

48

49 **INTRODUCTION**

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

64 Identification of species implicated in food poisoning is important to properly treat patients, as  
65 the symptoms and appropriate treatment vary depending on the species involved <sup>2</sup>.

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

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

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

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

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

## 93 **METHODS**

### 94 **Construction of the MSI reference database.**

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

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

106 Each carpophore was also submitted for DNA sequence analysis of the ITS1-ITS2 region using  
107 the ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-  
108 3') primers, according to the protocol described by Fujita et al.<sup>28</sup>, with an annealing temperature  
109 of 55°C. Identification at the species level was confirmed using a phylogenetic tree and the  
110 reference identifications published by Cui et al. in 2020<sup>29</sup> (supplemental figure 1).

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

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

138 **Score determination**

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

145 **Identification threshold**

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

153 **Identification scores per portion**

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

161 **Performance tests**



162 1/ Using the MSI application, we compared the database against a validation sample of 9  
163 carpophores (stem portions only) belonging to the same mycelium (i.e., collected at the same  
164 time and in the same location, belonging to the same fairy ring) as the carpophores used for the  
165 reference database. The validation sample corresponded to 6 *Amanita* species: *A. citrina*, *A.*  
166 *muscaria*, *A. ovoidea*, *A. pantherina*, *A. phalloides* and *A. rubescens*.

167 2/ Using the MSI application, we also compared the database against an external test sample of  
168 44 carpophores (73 portions) collected during a six-year period from four regions in France (32  
169 samples from the Vosges Forest between 2013 and 2019, 1 sample from Provence in 2016, 8  
170 samples from the Paris area in 2019 and 3 samples from the Doubs Forest in 2017).

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

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

## 182 **RESULTS**

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

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

#### 195 **Identification threshold.**

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

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

#### 201 **Identification scores per portion.**

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

#### 209 **Identification results.**

210 Identification of carpophores from the same mycelium.

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

215 Identification of carpophores from different mycelia.

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

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

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

230 Decay test.

231 Two *A. phalloides* carpophores were tested over the course of three consecutive days until the  
232 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<sup>4,31</sup>. 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<sup>15</sup>.

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<sup>4,8,32</sup>.

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<sup>27</sup>. While testing the database by comparing the reference spectra

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

261 After defining the identification threshold, we submitted 36 spectra of carpophores belonging to  
262 the same mycelia as references for the MSI application. When considering an identification  
263 threshold of 20, identification performance was excellent with scores reaching a maximum of  
264 76.3. When considering only the best identification results of the four spectra submitted for each  
265 mushroom sample, 100% of the samples were correctly identified.

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

270 The majority of misidentifications (7 carpophores) were associated with the section *Vaginatae*,  
271 which comprises a large number of species that were not included in our reference database, and  
272 which is continuously evolving. In 2009, Neville and Poumarat <sup>33</sup> described over 50 species in  
273 this subsection, while new species have been described since then by other authors <sup>29,34,35</sup>. In  
274 2020, Hanss and Moreau compiled a total of 86 species in the *Vaginatae* section, using ITS DNA  
275 sequencing <sup>36</sup>, and there are currently 96 provisional names recorded by Tulloss and Yang <sup>37</sup> in  
276 this section. Species-level identification of carpophores from the *Vaginatae* section using DNA  
277 databases can also be challenging, as new species have recently been phenotypically described  
278 and DNA sequences are not always available. However, when this subsection was excluded, 95%  
279 (35/37) of the carpophores and 85% (52/61) of the portions were correctly identified.

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

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

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

303

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315 **Conflicts of Interest : None**

316

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434 **FIGURES LEGENDS**

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

437

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

439

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

441

442 **Figure 4:** Score distribution for the various decay days (white boxes: spectra realized at decay  
443 day 1; gray boxes: spectra realized at decay day 2; black boxes: spectra realized at decay day 3).

444 The lower and upper portions of the box represent the lower and upper quartiles, respectively.

445 The line represents the median value. The ends of the whiskers represent the lowest datum  
446 included in the 1.5 interquartile range of the lower quartile and the highest datum included in the

447 1.5 interquartile range of the upper quartile. Outlier values are represented by circles. For each

448 spectrum, scores noted correspond to the identification against the same specimen but at different

449 decay days. Therefore, for each spectrum, three scores were used: decay day 1, decay day 2 and

450 decay day 3.

451 **Supplemental Figure 1:** Phylogeny using ITS sequences of the specimens collected in this

452 study, using the maximum likelihood (ML) analysis, and 1000 bootstraps. Bootstrap values are

453 represented by stars, and values below 70 are not shown. Sequences from Cui et al. <sup>29</sup> are

454 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
<i>A. caesarea</i>	Provence (1)	MW589070	1 / 10	1 / 10	1 / 10	1 / 10	4 / 40	2013 (1)	No
<i>A. citrina</i>	Provence (6)  Vosges (1)	MW589071* MW589072 MW589073 MW589074 MW589075	7 / 52	7 / 52	0 / 0	2 / 20	16 / 124	2013 (2), 2014 (1), 2015 (1), 2016 (3)	No
<i>A. intermedia</i>	Provence (1)	MW589076	1 / 10	1 / 10	0 / 0	0 / 0	2 / 20	2013 (1)	No
<i>A. junquillea</i>	Provence (3)	MW589077 MW589078*	3 / 18	3 / 18	0 / 0	0 / 0	6 / 36	2014 (1), 2016 (2)	No
<i>A. muscaria</i>	Provence (3)  Bordeaux (1)  Vosges (1)	MW589079 MW589080 MW589081 No sequence available MW589082	4 / 34	5 / 44	1 / 4	1 / 10	11 / 92	2013 (2), 2014 (1), 2015 (1), 2016 (1)	No
<i>A. ovoidea</i>	Provence (2)	No sequence available	2 / 19	2 / 20	0 / 0	0 / 0	4 / 39	2013 (2)	No
<i>A. pantherina</i>	Provence (1) Bordeaux (2)	MW589083 MW589126*	3 / 18	3 / 18	0 / 0	0 / 0	6 / 36	2013 (1), 2016 (2)	No
<i>A. phalloides</i>	Provence (4)   Bordeaux (2)	MW589084 MW589085 MW589086 MW589087 MW589127*	10 / 88	10 / 88	6 / 60	0 / 0	26 / 236	2013 (3), 2014 (1), 2016 (2)	Day 1, day 2 and day 3
<i>A. porphyria</i>	Provence (3)	MW589088*	3 / 12	3 / 12	0 / 0	0 / 0	6 / 24	2016 (3)	No
<i>A. proxima</i>	Provence (1)	MW589089	1 / 10	1 / 10	0 / 0	0 / 0	2 / 20	2013 (1)	No
<i>A. rubescens</i>	Provence (2)  Bordeaux (2)	MW589091 MW589092 MW589128*	4 / 28	4 / 28	1 / 4	0 / 0	9 / 60	2014 (2), 2016 (2)	No
<i>A. spissa</i>	Provence (1)	MW589090	1 / 10	1 / 10	0 / 0	0 / 0	2 / 20	2013 (1)	No
<i>A. submembranacea</i>	Provence (1) Vosges (1)	MW589094 MW589093	2 / 14	2 / 14	0 / 0	0 / 0	4 / 28	2015 (1), 2016 (1)	No
<i>A. verna var decipiens</i>	Provence (2)	MW589095	2 / 20	2 / 20	0 / 0	0 / 0	4 / 40	2016 (2)	No

456

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

Portion spectra	Reference portion	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_ <i>A. muscaria</i>	stem	<i>Amanita muscaria</i>	55.26	Correct
Provence2013-M127_ <i>A. citrina</i>	stem	<i>Amanita citrina</i>	76.3	Correct
Provence2013-M132_ <i>A. citrina</i>	stem	<i>Amanita citrina</i>	65.53	Correct
Provence2013-M004_ <i>A. intermedia</i>	stem	<i>Amanita intermedia</i>	42.63	Correct
Provence2013-M015_ <i>A. muscaria</i>	stem	<i>Amanita muscaria</i>	29.28	Correct
Provence2013-M166_ <i>A. pantherina</i>	stem	<i>Amanita pantherina</i>	54.42	Correct
Provence2013-M171_ <i>A. rubescens</i>	stem	<i>Amanita rubescens</i>	53.3	Correct
Provence2013-M174_ <i>A. ovoidea</i>	stem	<i>Amanita ovoidea</i>	29.3	Correct
Provence2013-M180_ <i>A. phalloides</i>	stem	<i>Amanita phalloides</i>	47.15	Correct

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

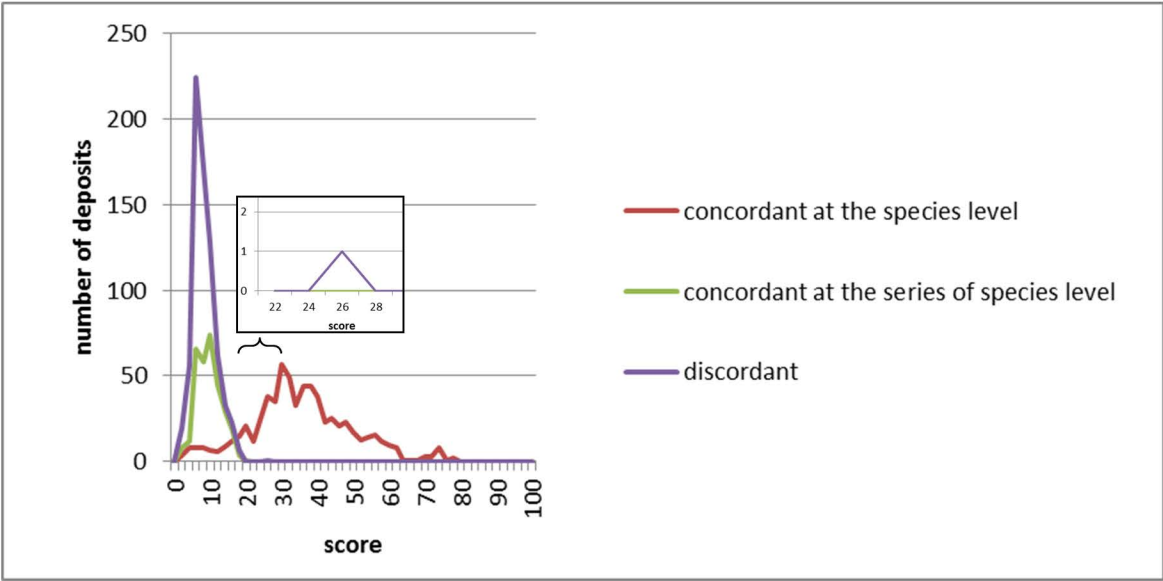
Sample-DNA identification	NCBI accession number for ITS sequences	Portion	MSI identification	Maximum score	Validation
Doubs2017-A_A. <i>citrina</i>	No sequence available	stem	<i>Amanita citrina</i>	33.9	Correct
Doubs2017-B_A. <i>muscaria</i>	No sequence available	stem	<i>Amanita muscaria</i>	28.6	Correct
Doubs2017-C_A. <i>rubescens</i>	No sequence available	stem	<i>Amanita rubescens</i>	25.3	Correct
Paris2019-A_A. <i>citrina</i>	MW589096	ring	<i>Amanita citrina</i>	21.1	Correct
		stem	<i>Amanita citrina</i>	23.2	Correct
		gill	<i>Amanita citrina</i>	30.4	Correct
		volva	Unidentified	0	No identification
Paris2019-B_A. <i>af. Vaginatae section</i>	No ITS sequence available	stem	Unidentified	0	No identification
		gill	Unidentified	0	No identification
Paris2019-C_A. <i>muscaria</i>	MW589097	stem	<i>Amanita muscaria</i>	48.2	Correct
		gill	<i>Amanita muscaria</i>	52.1	Correct
Paris2019-D_A. <i>pantherina</i>	No ITS sequence available	stem	Unidentified	0	No identification
		gill	<i>Amanita pantherina</i>	33.3	Correct
Paris2019-E_A. <i>A. rubescens</i>	No ITS sequence available	ring	<i>Amanita rubescens</i>	31.4	Correct
		stem	<i>Amanita rubescens</i>	30.1	Correct
		gill	<i>Amanita rubescens</i>	37.2	Correct
Paris2019-F_A. <i>submaculata</i>	MW589098	stem	Unidentified	0	No identification
		gill	Unidentified	0	No identification
Paris2019-G_A. <i>rubescens</i>	MW589099	stem	<i>Amanita rubescens</i>	32.3	Correct
		gill	<i>Amanita rubescens</i>	32.4	Correct
Paris2019-H_A. <i>citrina</i>	No sequence available	stem	<i>Amanita citrina</i>	36.8	Correct
		gill	<i>Amanita citrina</i>	49.1	Correct
Provence2016-M338_A. <i>muscaria</i>	MW589100	stem	<i>Amanita muscaria</i>	46.7	Correct
		gill	<i>Amanita muscaria</i>	46.4	Correct
Vosges2013-C_A. <i>rubescens</i>	MW589101	stem	Unidentified	26.9	Correct
		gill	<i>Amanita rubescens</i>	31.5	Correct
Vosges2013-D_A. <i>fulva</i>	MW589102	stem	Unidentified	0	No identification
		gill	Unidentified	0	No identification
Vosges2013-E_A. <i>muscaria</i>	MW589103	stem	<i>Amanita muscaria</i>	52.9	Correct
		gill	<i>Amanita muscaria</i>	43.7	Correct
Vosges2013-F_A. <i>citrina</i>	MW589104*	stem	<i>Amanita citrina</i>	40.7	Correct
		gill	<i>Amanita citrina</i>	46.5	Correct
		gill	<i>Amanita citrina</i>	58.4	Correct
Vosges2013-F1_A. <i>citrina</i>	MW589104*	gill	<i>Amanita citrina</i>	43.6	Correct
Vosges2013-F2_A. <i>citrina</i>		stem	<i>Amanita citrina</i>	23.1	Correct
Vosges2013-G_A. <i>citrina</i>	MW589105*	gill	<i>Amanita citrina</i>	457	Correct
		gill	<i>Amanita citrina</i>	30.5	Correct
		gill	Unidentified	29.3	Correct
Vosges2013-H1_A. <i>junquillea</i>	MW589106	stem	Unidentified	0	No identification
		gill	<i>Amanita junquillea</i>	21.6	Correct
Vosges2013-H2_A. <i>citrina</i>	MW589107	stem	Unidentified	36.1	Correct
		gill	<i>Amanita citrina</i>	19	No identification
Vosges2017-A_A. <i>citrina</i>	MW589108	gill	<i>Amanita citrina</i>	49.3	Correct
Vosges2017-B_A. <i>citrina</i>	MW589109	gill	<i>Amanita citrina</i>	46.6	Correct
Vosges2017-C_A. <i>citrina</i>	MW589110	gill	<i>Amanita citrina</i>	42	Correct
Vosges2017-D_A. <i>muscaria</i>	MW589111	gill	<i>Amanita muscaria</i>	46.7	Correct
Vosges2017-E_A. <i>muscaria</i>	MW589112	gill	<i>Amanita muscaria</i>	55	Correct
Vosges2017-F_A. <i>muscaria</i>	MW589113	gill	<i>Amanita muscaria</i>	48.1	Correct
Vosges2017-G_A. <i>fulva</i>	MW589114	gill	Unidentified	0	No identification

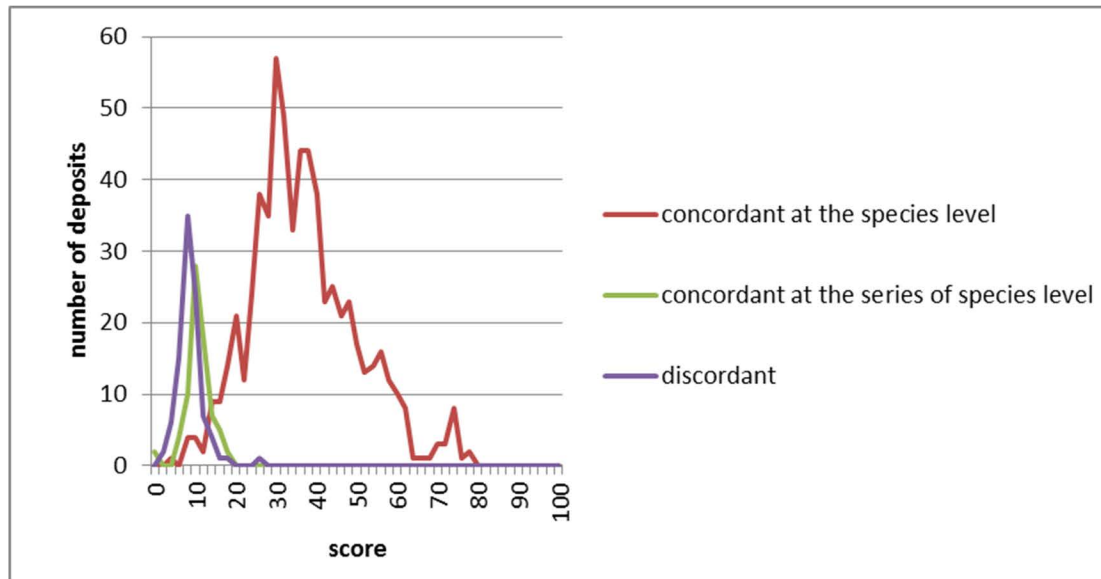
Vosges2017-H_A. <i>umbrinolutea</i>	MW589115	gill	Unidentified	0	No identification
Vosges2017-I_A. <i>rubescens</i>	No sequence available	gill	<i>Amanita rubescens</i>	36.9	Correct
Vosges2017-J_A. <i>rubescens</i>	No sequence available	gill	<i>Amanita rubescens</i>	39.8	Correct
Vosges2017-K_A. <i>rubescens</i>	No sequence available	gill	<i>Amanita rubescens</i>	28.5	Correct
Vosges2019-A_A. <i>fulva</i>	MW589116	stem	Unidentified	0	No identification
		gill	Unidentified	0	No identification
Vosges2019-B_A. <i>rubescens</i>	MW589117	stem	<i>Amanita rubescens</i>	18.3	No identification
		gill	<i>Amanita rubescens</i>	41.3	Correct
Vosges2019-C_A. <i>fulva</i>	MW589118	stem	Unidentified	0	No identification
		gill	Unidentified	0	No identification
Vosges2019-D_A. <i>rubescens</i>	MW589119	stem	Unidentified	17.1	No identification
		gill	<i>Amanita rubescens</i>	19.3	No identification
Vosges2019-E_A. <i>citrina</i>	MW589120	stem	<i>Amanita citrina</i>	31.7	Correct
		gill	<i>Amanita citrina</i>	39.2	Correct
Vosges2019-F_A. <i>citrina</i>	MW589121	stem	<i>Amanita citrina</i>	45	Correct
		gill	<i>Amanita citrina</i>	49.3	Correct
Vosges2019-G_A. <i>fulva</i>	MW589122	stem	Unidentified	0	No identification
		gill	Unidentified	0	No identification
Vosges2019-H_A. <i>muscaria</i>	MW589123	stem	<i>Amanita muscaria</i>	47.9	Correct
		gill	<i>Amanita muscaria</i>	44	Correct
Vosges2019-I_A. <i>rubescens</i>	MW589124	stem	<i>Amanita rubescens</i>	24.8	Correct
		gill	<i>Amanita rubescens</i>	40.3	Correct
Vosges2019-J_A. <i>rubescens</i>	MW589125	stem	<i>Amanita rubescens</i>	35.7	Correct
		gill	<i>Amanita rubescens</i>	34.7	Correct

473

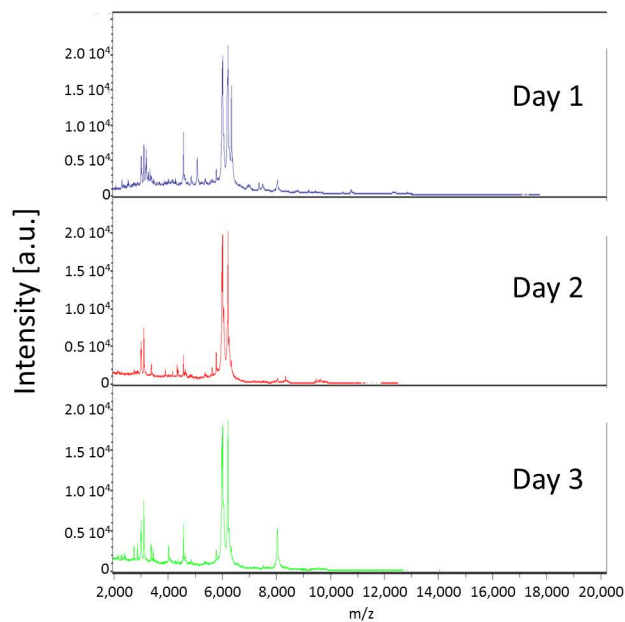
474 \*several different carpophores from the same mycelium incorporated as references, only one  
475 sequenced.



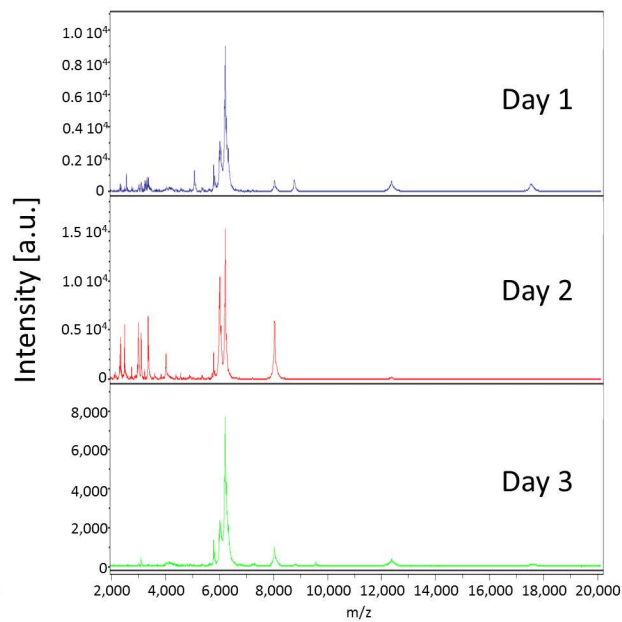




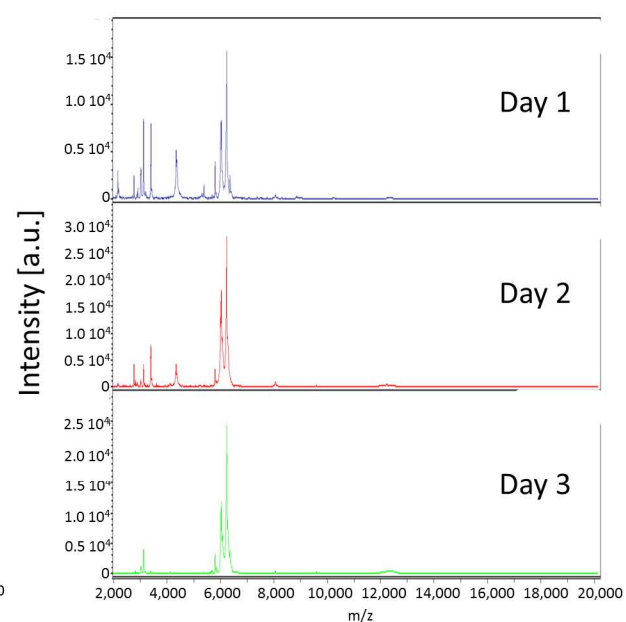
*Amanita phalloides* M150 Stem portion



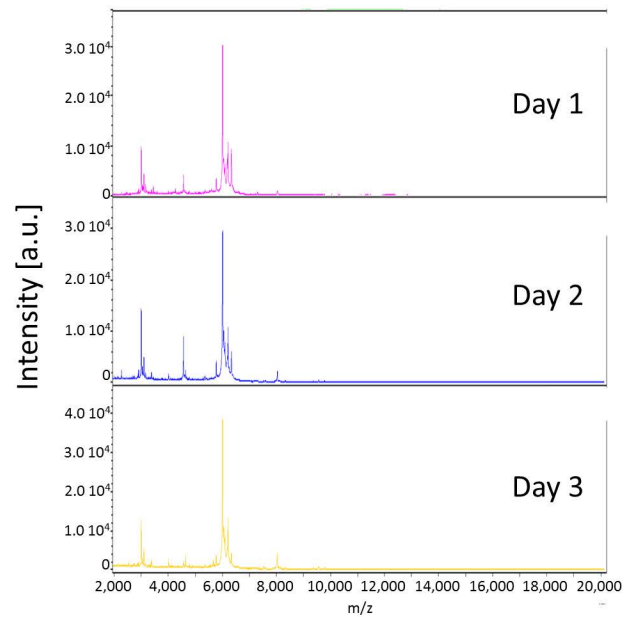
*Amanita phalloides* M150 Gills portion



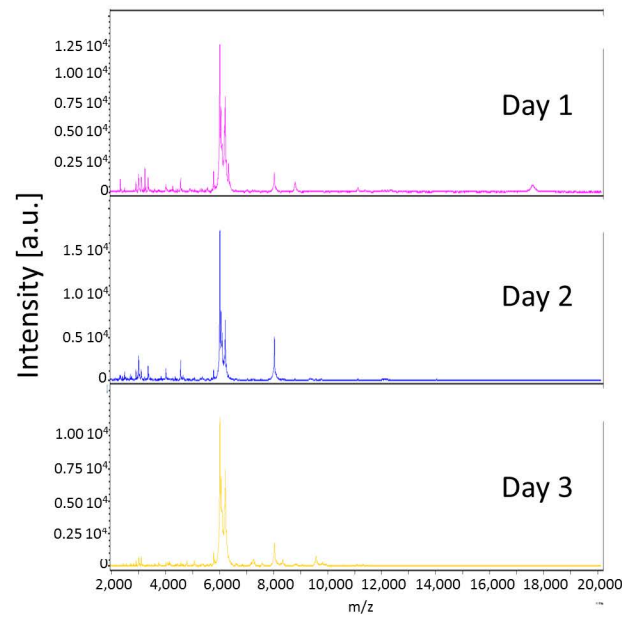
*Amanita phalloides* M150 Volva portion



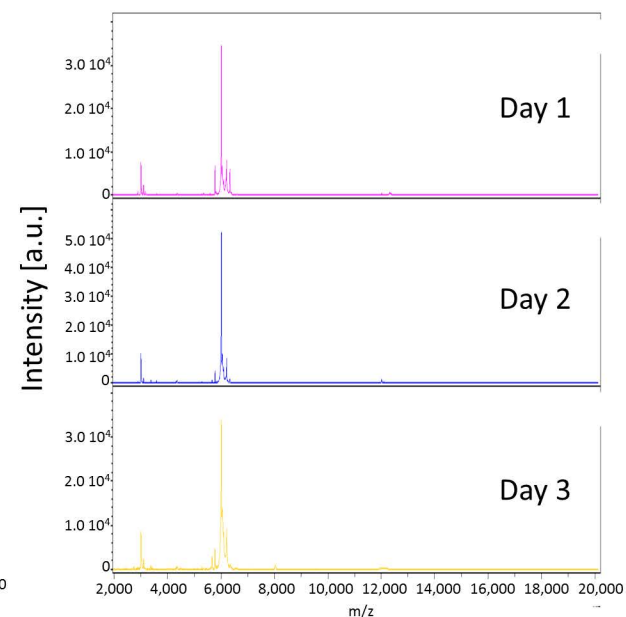
*Amanita phalloides* M160 Stem portion

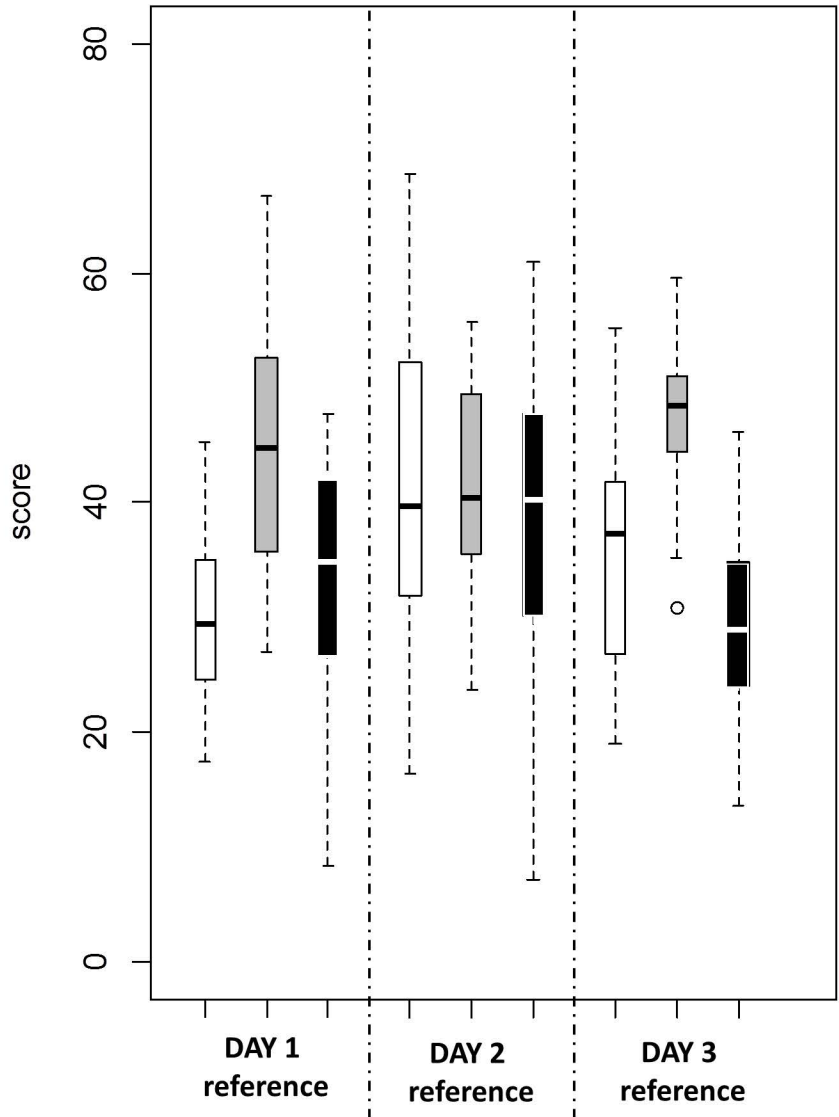


*Amanita phalloides* M160 Gills portion



*Amanita phalloides* M160 Volva portion





- Species**
- A.caesarea*
  - A.citrina*
  - A.intermedia*
  - A.porphyrina*
  - A.muscaria*
  - A.pantherina*
  - A.junquillea*
  - A.rubescens*
  - A.spissa*
  - A.submaculata*
  - A.phalloides*
  - A.verna*
  - A.virosa*
  - A.fulva*
  - A.submembranacea*
  - A.umbrinolutea*
  - A.proxima*

