



## 22 ABSTRACT

23 **Background:** Differentiation of the species within the *Cryptococcus neoformans* complex (*C.*  
24 *deneoformans*, *C. neoformans* and *C. neoformans* interspecies hybrids –*C. deneoformans* x *C.*  
25 *neoformans*-) is important to define the epidemiology of the infection.

26 **Objectives:** In this study we attempted the discrimination of three *C. neoformans* species using  
27 MALDI-TOF MS coupled with an in-house library.

28 **Methods:** All *Cryptococcus* spp. isolates were identified by AFLP markers. An in-house database  
29 was constructed 26 well characterized *C. deneoformans*, *C. neoformans* and interspecies hybrids.  
30 Forty-four *Cryptococcus* spp. isolates were blindly identified using MALDI-TOF MS (Bruker  
31 Daltonics) and the expanded library. Their protein spectra were also submitted to hierarchical  
32 clustering and the resulting species were verified via Partial Least Squares Differential Analysis  
33 (PLS-DA) and Support-Vector Machine (SVM).

34 **Results:** MALDI-TOF MS coupled with the in-house library allowed 100% correct identification of  
35 *C. deneoformans* and *C. neoformans* but misidentified the interspecies hybrids. The same level of  
36 discrimination among *C. deneoformans* and *C. neoformans* was achieved applying SVM. The  
37 application of the PLS-DA and SVM algorithms in a two-step analysis allowed 96.95% and 96.55%  
38 correct discrimination of *C. neoformans* from the interspecies hybrids, respectively. Besides, PCA  
39 analysis prior to SVM provided 98.45% correct discrimination of the 3 species analysed in a one-  
40 step analysis.

41 **Conclusions:** Our results indicate that MALDI-TOF MS could be a rapid and reliable tool for the  
42 correct discrimination of *C. deneoformans* and *C. neoformans*. The correct identification of the  
43 interspecies hybrids could only be achieved by hierarchical clustering with other protein spectra  
44 from the same species.

45 **Key Words:** *Cryptococcus* spp, MALDI-TOF MS, in-house library, AFLP, hierarchical clustering

## 46 INTRODUCTION

47 The genus *Cryptococcus* has classically comprised two sibling species with great  
48 importance from the clinical point of view: *Cryptococcus neoformans* and *C. gattii*, the causative  
49 agents of cryptococcosis. Whilst *C. neoformans* complex has been associated with meningitis in  
50 immunosuppressed patients, *C. gatti* has been shown to cause disease in both immune competent  
51 and immunocompromised population<sup>1,2</sup>. Species differentiation is important in order to establish the  
52 epidemiology, virulence and susceptibility pattern to the commonly used antifungal drugs<sup>3-6</sup>. So far,  
53 species assignment is achieved by morphology analysis of the colonies grown on specific culture  
54 media and serological tests<sup>7</sup>. The availability of DNA-based methodologies as restriction fragment  
55 length polymorphism (RFLP) analysis<sup>8</sup>, amplified fragment length polymorphism (AFLP) analysis<sup>9</sup>,  
56 multilocus microsatellite typing -MLMT<sup>-10</sup>, and multilocus sequence typing –MLST<sup>-11</sup> has allowed  
57 the identification of *Cryptococcus* species and molecular types in the last years<sup>8-13</sup>. Genotyping  
58 methods have identified the following major molecular types: AFLP1/VNI, AFLP1A,  
59 AFLP1B/VNII for *C. neoformans*; AFLP2/VNIV for *C. deneoformans*, AFLP3/VNIII for the  
60 interspecies hybrid *C. neoformans neoformans* x *C. deneoformans*; and AFLP4/VGI, AFLP5/VGIII,  
61 AFLP6/VGII, AFLP7/VGIV and AFLP10/VGIV, VGII for *C. gattii* complex<sup>14,15</sup>.

62 Molecular techniques have shown to be accurate and robust although the whole procedure  
63 is cumbersome, time consuming, and delays the final identification. Although genomic analysis is  
64 currently the gold standard for *Cryptococcus* identification, its high requirements in hands-on time  
65 and expertise has led to the evaluation of alternative tools.

66 Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF  
67 MS) has emerged as a promising technology for the rapid and reliable identification of yeasts<sup>16-18</sup>.  
68 Isolates belonging to the *Candida* genus have been shown to be easily identified at the species level

69 either from single colonies or directly from clinical samples using MALDI-TOF MS<sup>19</sup>. However,  
70 non-Candida yeasts still represent a challenge for this technology, especially when trying to identify  
71 genera poorly represented or even lacking in the commercial databases<sup>20</sup>. In this case, expanded in-  
72 house databases containing protein spectra from the underrepresented species and genera have  
73 shown to overcome this drawback<sup>16</sup>. Although this approach has worked before for the  
74 discrimination between *C. neoformans* and *C. gatti* complexes<sup>21,22</sup>, the available information about  
75 MALDI-TOF discrimination within the *C. neoformans* complex is still limited<sup>23</sup>.

76 In this study, MALDI-TOF has been applied for the discrimination between *C.*  
77 *deneoformans*, *C. neoformans* and the interspecies hybrids. For this purpose, two approaches have  
78 been applied: a database was built using well-characterized isolates and automated peak analysis  
79 was performed.

80

## 81 **MATERIALS AND METHODS**

### 82 **Isolates and molecular identification**

83 We retrospectively selected 70 *Cryptococcus* spp. isolates from clinical samples (n=70)  
84 belonging to 67 patients admitted to Hospital Gregorio Marañón (Madrid, Spain) from 1994 to  
85 2007. Isolates sourced from cerebrum spinal fluids (51%), blood (33%), respiratory samples (10%),  
86 and others (6%). They were morphologically identified on Columbia agar + 5% sheep blood plates  
87 (Biomérieux Marcy L'étoile, France) at 35 ° C, and by means of the ID 32C system (bioMérieux,  
88 Marcy l'Etoile, France). All isolates were stored at -80°C in water until further analysis. All isolates  
89 were previously identified by AFLP analysis<sup>24</sup> and were stored at -80°C in water until further  
90 analysis. Molecular identifications were considered as the reference in our study.

91

## 92 **Database construction**

93 Twenty-six *Cryptococcus* isolates - *C. neoformans* (n=12), interspecies hybrids (n=10) and  
94 *C. deneoformans* (n=4)- were processed according to the manufacturer's instructions and added to  
95 the in-house database (HGM library) as individual Main Spectra (MSPs).

96 The procedure for adding new entries to an in-house library has already been described<sup>25</sup>.  
97 Briefly, the instrument was calibrated before spectra acquisition using freshly prepared BTS;  
98 *Cryptococcus* isolates were processed as explained below and then spotted onto eight positions in  
99 the MALDI target plate and each position was read three times. Twenty-four protein spectra were  
100 thus achieved, 20 of which had to be identical in order to be accepted by the software (Biotyper,  
101 Bruker Daltonics, Bremen, Germany) as a MSP and added to the extended library.

## 102 **MALDI-TOF identification**

103 Forty-four *Cryptococcus* spp. isolates were blindly analysed using an LT Microflex  
104 benchtop MALDI-TOF mass spectrometer (Bruker Daltonics) for spectra acquisition, using default  
105 settings. For the identification of the protein spectra, the updated BDAL database containing 8223  
106 MSPs (Bruker Daltonics) was applied. This database contains 12 reference MSPs from *C.*  
107 *neoformans* and 7 from *C. deneoformans*. Besides, the expanded in-house HGM library developed  
108 in this study was used in combination with the commercial database.

109 The sample processing method applied consisted of a mechanical disruption step followed  
110 by a standard protein extraction. Briefly, a few colonies were picked, re-suspended in 300 µl water  
111 HPLC-grade and 900µl ethanol, and submitted to 5 min vortexing. After a brief spin, the  
112 supernatant was discarded and the pellet allowed drying completely at RT. Protein extraction with  
113 formic acid and acetonitrile was performed and 1µl of the supernatant was spotted onto the MALDI

114 target plate in duplicates. Once the spots were dry, they were covered with 1µl HCCA matrix  
115 (Bruker Daltonics), prepared following the manufacturer’s instructions (Figure 1).

116 The identifications provided by MALDI-TOF MS were compared at the species level with  
117 those provided by AFLP analysis regardless of their score value (Table 1). Besides, score values  
118  $\geq 2.0$  were considered as “high-confidence” scores and those  $\geq 1.7$  as “low-confidence” ones. Score  
119 values below 1.6 were only considered when consistent over the four top identifications, otherwise  
120 they were considered as “not reliable”.

### 121 **Peak Analysis**

122 For the classification of the three species of *Cryptococcus* their protein spectra were  
123 processed using Clover MS Data Analysis software (Clover Biosoft, Granada, Spain) with the  
124 parameters shown in Table S1 in order to achieve a peak matrix with a representative mass list in  
125 the range 2400m/z to 12000m/z. Furthermore, spectra alignment was performed. First, the replicates  
126 from the same isolate were aligned in order to get an average spectrum. Finally, all average spectra  
127 were aligned together.

128 The rate of presence for the biomarker peaks was calculated for each species and then  
129 compared among species. Receiver Operating Characteristic (ROC) curve with Area under the  
130 Curve –AUC- higher than 0.99 were used as quality indicators to measure the sensibility and  
131 specificity of a selected biomarker.

132 Once the putative biomarkers were selected and analysed, a peak matrix was built  
133 containing all the aligned spectra from all *Cryptococcus* isolates, processed as described in Table  
134 S2. This peak matrix was constructed with ten species-specific biomarkers and it was used as input  
135 for a dendrogram obtained measuring Euclidean distance from Principal Component Analysis  
136 (PCA) scores.

137 Over the peak matrix, two approaches were applied in order to discriminate the three  
138 *Cryptococcus* species. The first one was a two-step method in which the discrimination of *C.*  
139 *deneoformans* from the other two species was performed as a first step and it was replicated by  
140 means of two machine learning algorithms on the same peak matrix: supervised PLS-DA and SVM.  
141 Results were validated using k-fold cross validation method. In the second step, a new peak matrix  
142 was performed in order to achieve a better discrimination of *C. neoformans* from the interspecies  
143 hybrids. A second dendrogram was performed using the above mentioned parameters. Again, PLS-  
144 DA and SVM was performed to this second peak matrix to replicate the classification and the k-fold  
145 cross validation method was applied. The two-step method was further improved by the exclusion  
146 from the peak matrix of peaks that did not provide enough discrimination.

147 In order to simplify the workflow, a one-step method was assayed so that the capacity of  
148 the algorithms to discriminate the three *Cryptococcus* species at the same time was tested. In this  
149 case, only one peak matrix with spectra from the three species was built and 5 species-specific  
150 biomarkers were included. The alignment and processing parameters were the same as in the two-  
151 steps approach. The one-step method was evaluated using the peak matrix generated as input data  
152 for PLS-DA analysis and SVM analysis. Besides, the validation in both cases was performed using  
153 k-fold confusion matrix.

#### 154 **Ethic Statement**

155 The hospital Ethics Committee approved this study and gave consent for its performance  
156 (Code: MICRO.HGUGM.2017-003). Since only microbiological samples were analysed, not  
157 human products, all the conditions to waive the informed consent have been met.

158

#### 159 **RESULTS**

160 Genotyping of the isolates detected three different genotypes. The most common genotype  
161 was AFLP1/1B (*C. neoformans*, n=34; 49%), followed by AFLP3 (interspecies hybrids, n=29;  
162 41%) and AFLP2 (*C. deneoformans*, n=7; 10%).

163 The application of MALDI-TOF MS and the commercial database allowed the correct  
164 identification of 18/22 *C. neoformans* isolates (81.8%) and 1/3 *C. deneoformans* isolates (33.3%);  
165 the remaining *C. neoformans* isolates –n=4– could not be reliably identified and for 2 *C.*  
166 *deneoformans* isolates MALDI-TOF did not provide the species identification (Table 1). The  
167 identification of the interspecies hybrids (n=19) was not achieved using the commercial database  
168 due to the lack of representation of this microorganism. These isolates were identified as *C.*  
169 *neoformans* complex in 9 cases (score $\geq$ 2.0, n=7; score $>$ 1.7, n=1; score $<$ 1.6, n=1), as *C.*  
170 *deneoformans* in 7 cases (score $>$ 1.7, n=4; score $<$ 1.6, n=3) and as *C. neoformans* in 3 cases  
171 (score $>$ 1.7) –Table 1-.

172 Only two isolates (8.0%) were correctly identified at the species level with high-confidence  
173 score values ( $\geq$ 2.0) whilst 52.3% of the samples were identified with low-confidence scores ( $>$ 1.7) -  
174 Table 1-. Another 4 isolates were reliably identified to the species level, although with scores values  
175 ranging between 1.7 and 1.6 and, finally, 8 isolates obtained scores below 1.6. The latter can be  
176 considered as unreliable identifications.

177 Using the in-house library all *C. neoformans* and *C. deneoformans* isolates were correctly  
178 identified by MALDI-TOF MS at the species level (Table 1). Moreover, 21/25 isolates (84.0%)  
179 were identified with score values  $\geq$ 2.0 which indicates a high-confidence level. The reliability of the  
180 identification was further demonstrated by the fact that the top 4-5 identifications were identical in  
181 all cases. In all but two cases these top reference isolates belonged to the HGM in-house library.

182 However, the implementation of the expanded HGM library only allowed the correct  
183 identification of 12/19 interspecies hybrids, 7 of them with score values above 2.0. The high



184 closeness of the interspecies hybrids with the other two *Cryptococcus* species made it difficult for  
185 MALDI-TOF MS to discriminate among them and misidentified 7 interspecies hybrids as *C.*  
186 *neoformans* (Table 1).

187 To improve the identification of the interspecies hybrids and their discrimination from *C.*  
188 *deneoformans* and *C. neoformans*, peak analysis was performed. The search for species-specific  
189 biomarker peaks yielded a list of 10 peaks that allowed the differentiation of the *Cryptococcus*  
190 species analysed, with 5 of them showing higher discriminative power (Table 2). The two-step  
191 method allowed correct differentiation of the interspecies hybrids which clustered distinctly in the  
192 dendrograms built using two different hierarchical clustering variations (Figure 2 and Figure S2).  
193 These dendrograms showed three different clusters where *Cryptococcus deneoformans* isolates  
194 were clearly separated from *Cryptococcus neoformans* and the interspecies hybrids. Accurate  
195 differentiation among the 3 *Cryptococcus* species was achieved using the peak matrix built upon the  
196 5 most discriminative peaks, with only one spectrum from an interspecies hybrid misallocated in the  
197 *C. neoformans* cluster (Figure 2B). *C. neoformans* and the interspecies hybrids showed close  
198 relatedness between them based on their protein spectra.

199 The validation of the method yielded a k-fold (k=10) score of 96.92% for PLS-DA  
200 performed over the peak matrix with 10 biomarkers and 98.46% for the analysis with 5 biomarkers.  
201 However, SVM algorithm achieved 100% discrimination in both cases when PCA was applied  
202 (Table S3).

203 A second dendrogram was performed using hierarchical clustering analysis. It showed two  
204 well-defined clusters for *Cryptococcus neoformans* and the interspecies hybrids (Figure S2). In this  
205 step only the 3 biomarkers to differentiate *C. deneoformans* from interspecies hybrids were used  
206 (5453.91, 5552.90 and 7103.00 m/z). Furthermore, this second dendrogram was validated by PLS-  
207 DA and SVM algorithms. K-fold (k=10) was applied achieving 95.55% efficacy in both analyses.

208 In the single-step method, the peak matrix built with 5 biomarkers was used as an input for  
209 PLS-DA and SVM analysis in order to achieve the discrimination of the 3 *Cryptococcus* species  
210 simultaneously. PLS-DA analysis could not classify correctly the three varieties at the same time  
211 due to the low k-fold (k=10) values obtained. However, PCA performance prior to SVM allowed  
212 98.46% correct classification of the three *Cryptococcus* species (Figure 3). The efficacy of the  
213 method was tested by k-fold (k=10) cross validation analysis was above 95.0%. (Figure 3, Table  
214 S3)

215 As a result of this analysis, a visual method for the differentiation of the analyzed  
216 *Cryptococcus* species can be applied based on the presence of the 6688.67 m/z peak in the *C.*  
217 *neoformans* isolates and their absence in *C. deneoformans* isolates, where the peaks 6576.08 m/z  
218 and 7103.01 m/z could be detected. On the other hand, both sets of peaks are present in the  
219 interspecies hybrids although some of them (2842.14, 3084.11 and 8636.24 m/z) were detected in  
220 100% of the spectra from this species (Table 3). The visual detection of these biomarker peaks  
221 could provide a rapid and accurate identification of the *Cryptococcus* species prior to a more in-  
222 depth peak analysis using *ad-hoc* software.

223

## 224 **DISCUSSION**

225 Accurate identification of *Cryptococcus* species within the *Cryptococcus neoformans*  
226 complex provides valuable information about their epidemiology, sensitivity to commonly used  
227 antifungal drugs or virulence. Our results show that discrimination among the three *Cryptococcus*  
228 species analyzed—*C. deneoformans*, *C. neoformans* and interspecies hybrids- can be performed  
229 successfully using MALDI-TOF MS.

230 The implementation of the in-house database built in our laboratory allowed 100% correct  
231 species-level identification of the 25 *Cryptococcus deneoformans* and *C. neoformans* isolates used

232 to challenge it. Apart from the reliable identification of the analyzed *Cryptococcus* species, the in-  
233 house library also provided high confidence identifications in 63.6% of the cases (Table 1).  
234 Furthermore, these results showed consistency along the 10 top identifications provided by the mass  
235 spectrometry instrument, even for the hybrids. This fact is of great importance in the routine of the  
236 microbiology laboratory in order to transfer reliable information to the clinicians.

237 The results obtained are in agreement with those obtained by other authors<sup>21-23</sup>. However,  
238 the in-house library did not provide enough discrimination between the above-mentioned species  
239 and the interspecies hybrids. This goal was only fulfilled completely when peak analysis was  
240 performed and the three *Cryptococcus* species analyzed in this study distinctively clustered together  
241 (Figure 2). Other authors have provided species-level discrimination in 98.1-100% of the cases<sup>21, 23</sup>,  
242 <sup>26</sup>. Although some of these studies were performed on higher number of isolates, our results also  
243 reflect the improvements made on the commercial database during the last years.

244 The available commercial database has demonstrated to provide high species-level  
245 resolution for *C. deneoformans* and *C. neoformans*—76.0%— although score values <1.7 were  
246 obtained in 21.0% of the cases and species-level identification was not provided for 2 *C.*  
247 *deneoformans* isolates. These data supported the need of building expanded databases. However,  
248 even improvements in the reference databases proved not to be enough to differentiate the  
249 interspecies hybrids. This may be due to the algorithms used by the mass spectrometry instrument  
250 for species assignment and to the fact that the hybrids show peaks present of both parental species.  
251 Therefore, peak analysis using *ad-hoc* software was performed. A list of 10 biomarker peaks was  
252 achieved as the input for species classification (Table 2). The implementation of PLS-DA analysis  
253 in a two-step approach allowed the discrimination of *C. deneoformans* isolates in the first place and,  
254 subsequently, the correct classification of *C. neoformans* isolates and the interspecies hybrids in  
255 96.92% of the cases. Furthermore, the accuracy of this method increased when the number of  
256 biomarker peaks used was reduced to the five most discriminative ones (98.46%).

257           In order to simplify the analysis, a one-step method was proposed in order to classify the  
258 three species simultaneously. In this case, PLS-DA provided correct classification in less than  
259 75.0% of the cases but the application of SVM after PCA analysis allowed 96.92% correct  
260 discrimination of the analyzed isolates. This analysis provided a set of species-specific peaks for the  
261 *Cryptococcus* species within the *C. neoformans* complex that may be detected by visual inspection,  
262 representing a rapid and inexpensive approach for their discrimination.

263           In summary, our results demonstrate the usefulness of MALDI-TOF MS when applied in  
264 the microbiology laboratory for rapid and reliable identification of non-*Candida* yeasts. Although  
265 the updated commercial library provided correct species-level identification for a high number of *C.*  
266 *deneoformans* and *C. neoformans* isolates (43.2%), the identification of these species was missing  
267 or not reliable in 20.5% 18.2% of the cases, respectively. Moreover, the detection of the interspecies  
268 hybrids is not possible with the Biotyper database. However, the expanded in-house library allowed  
269 correct species-level identification for all *C. deneoformans* and *C. neoformans*, either by  
270 conventional identification with MALDI-TOF MS or by peak analysis (Figure 3). The interspecies  
271 hybrids required hierarchical clustering for their correct identification since their close relatedness  
272 with the other species made it difficult for MALDI-TOF to differentiate them from the other two  
273 species in a routine manner. This approach and the detection of species-specific peaks are  
274 recommended for the reliable discrimination of the three analyzed species.

275

## 276 **Conflict of Interest Statement**

277           The authors report no conflicts of interest. The authors alone are responsible for the content  
278 and the writing of the paper.

279

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288   and analysis, decision to publish, or preparation of the manuscript.

289

## 290 **Figure Legends**

291 **Figure 1.** Workflow of the sample preparation method used in this study to obtain proteins from  
292 *Cryptococcus* spp. isolates for their identification by MALDI-TOF MS.

293 **Figure 2.** Clustering of 65 *Cryptococcus* isolates included in this study. Five isolates could not be  
294 recovered from culture for further analysis.

295 **Figure 3.** Classification of the three *Cryptococcus* species by SVM in the one-step approach, using  
296 5 biomarker peaks.

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380

**Table 1.** Identification of *Cryptococcus neoformans*, *C. deneoformans* and interspecies hybrids using MALDI-TOF MS and the Biotyper library alone or in combination with the in-house HGM database. <sup>1</sup>Identified as *C. neoformans* complex (n=2); <sup>2</sup>Identified as *C. neoformans* complex (n=7); <sup>3</sup>Identified as *C. neoformans* complex (n=1), *C. deneoformans* (n=4) and *C. neoformans* (n=3); <sup>4</sup>Identified as *C. neoformans* complex (n=1) and *C. deneoformans* (n=3); <sup>5</sup>Identified as *C. neoformans* (n=7)

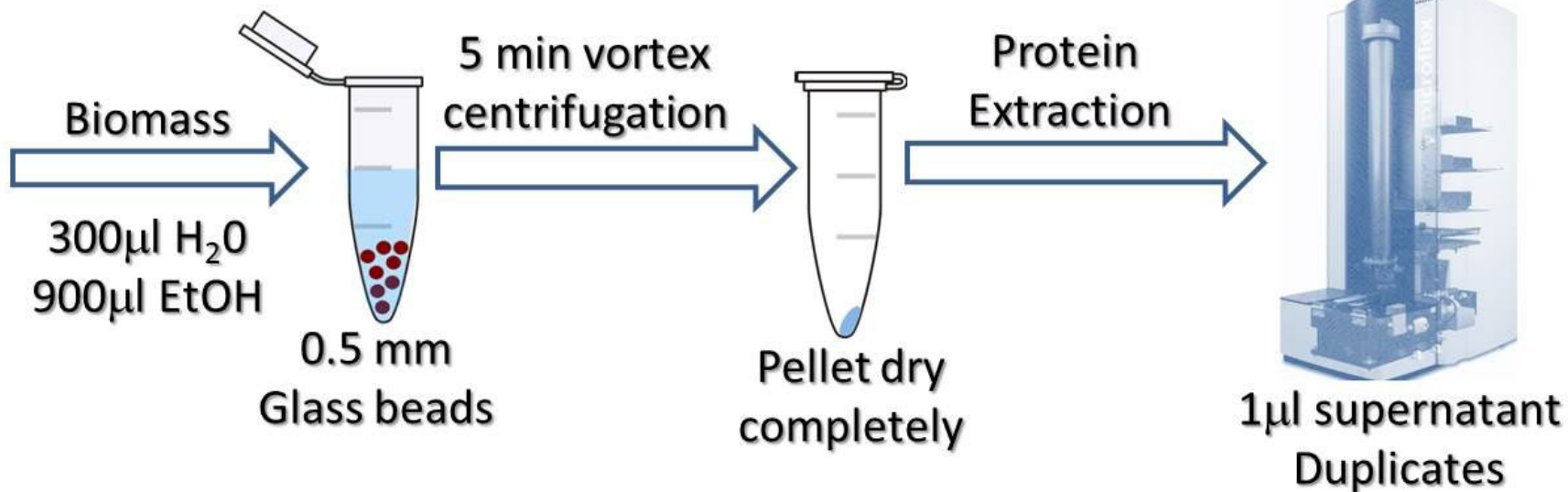
IDENTIFICATION BY DNA SEQUENCING	Isolates analyzed	<i>Cryptococcus spp. ISOLATES IDENTIFIED BY BIOTYPER WITH 8223 MSPs (%)</i>				<i>Cryptococcus spp. ISOLATES IDENTIFIED BY BIOTYPER 8223 MSPs + HGM LIBRARY (%)</i>		
		Score ≥2.0	Score ≥1.7	Score ≥1.6	Score <1.6	Score ≥2.0	Score ≥1.7	Score ≥1.6
<i>Cryptococcus neoformans</i>	22	2	13	3	4	18	4	0
<i>Cryptococcus deneoformans</i>	3	0	2 <sup>1</sup>	1	0	3	0	0
Interspecies hybrids	19	7 <sup>2</sup>	8 <sup>3</sup>	0	4 <sup>4</sup>	7	12 <sup>5</sup>	0
<b>TOTAL</b>	<b>44</b>	<b>9</b>	<b>23</b>	<b>4</b>	<b>8</b>	<b>28</b>	<b>16</b>	<b>0</b>

**Table 2.** List of the 10 representative mass peaks of *Cryptococcus* spp. Identified as potential biomarkers. These peaks were used for the construction of dendrograms and PLS-DA and SVM models. The 5 peaks marked with asterisks (\*) were selected for the simplified models. CV= Coefficient of Variation.

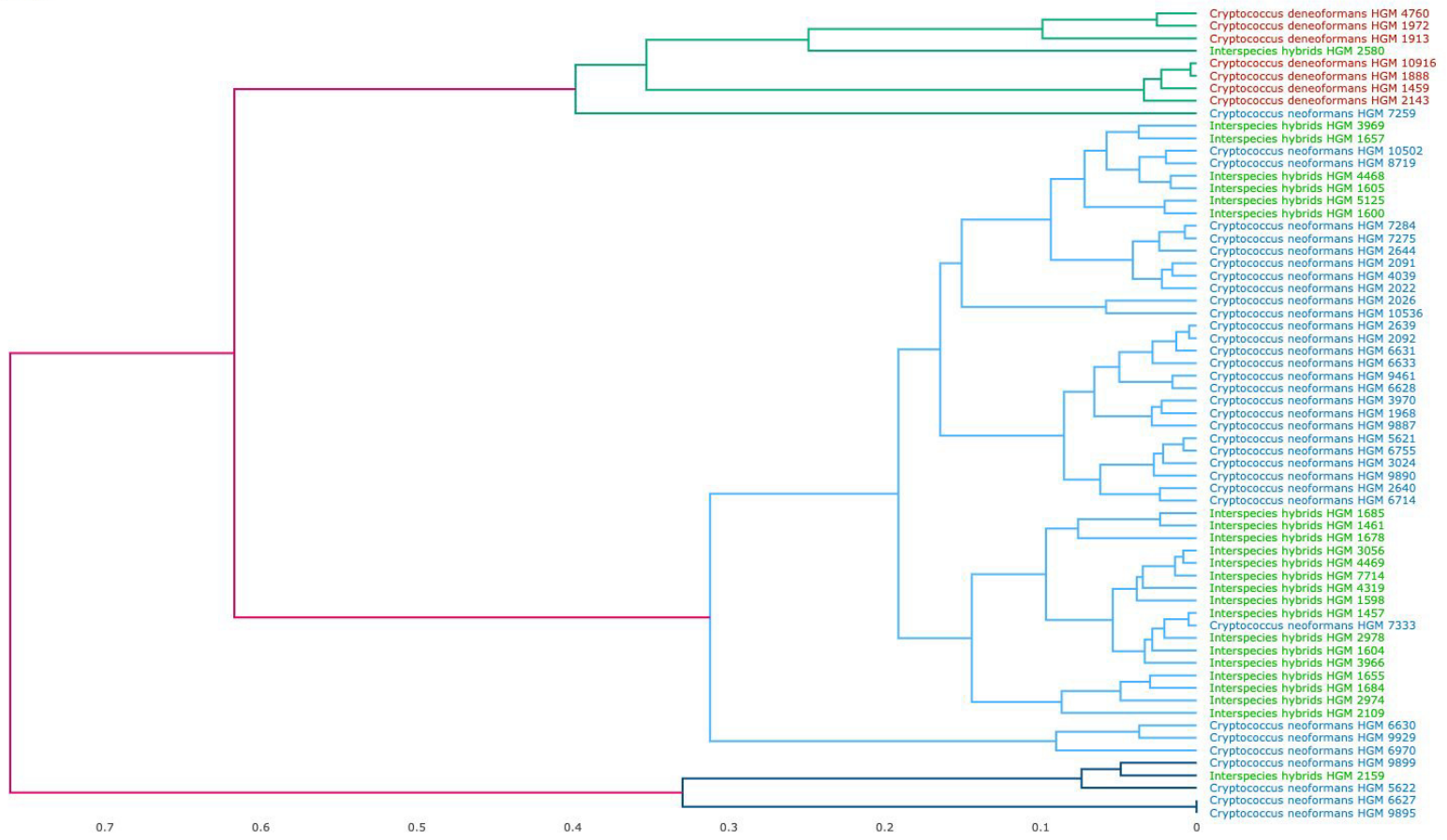
Mass (m/z)	Number of Spectra	<i>C. neoformans</i>	<i>C. neoformans</i> (CV)	<i>C. neoformans</i> (mean)	Interspecies hybrids	Interspecies hybrids (CV)	Interspecies hybrids (mean)	<i>C. deneoformans</i>	<i>C. deneoformans</i> (CV)	<i>C. deneoformans</i> (mean)
2488.07	54	30/34	88.749 %	4.401.767	24/24	69.617 %	2.811.789	0/7	-	-
2842.14	53	29/34	78.206 %	2.202.128	24/24	79.602 %	2.235.196	0/7	-	-
*3084.11	55	31/34	98.458 %	7800.06	24/24	89.283 %	5.969.393	0/7	-	-
*5453.91	27	1/34	0.0 %	72.906	23/24	65.081 %	731.902	3/7	12.654 %	748.872
*5552.90	27	1/34	0.0 %	558.307	23/24	73.624 %	1.418.905	3/7	47.3 %	2.763.278
6576.08	23	0/34	-	-	16/24	63.172 %	457.978	7/7	56.698 %	685.58
*6688.67	57	34/34	95.69 %	4.420.907	23/24	88.388 %	3.556.217	0/7	-	-
*7103.01	31	1/34	0.0 %	24.32	23/24	122.759 %	1.484.767	7/7	52.14 %	4.155.275
7830.42	18	0/34	-	-	11/24	46.494 %	719.13	7/7	39.831 %	449.704
8636.24	43	19/34	101.061 %	2.887.856	24/24	87.315 %	1.832.722	0/7	-	-

**Table 3.** Differentiation of the analyzed *Cryptococcus* species based on the absence/presence of biomarker peaks. Figures indicate the percentage (%) of isolates showing the indicated peak.

m/z	2842.14	3084.11	6576.08	6688.67	7103.01	8636.24
<i>C. deneoformans</i>	0	0	100	0	100	0
<i>C. neoformans</i>	85.3	91.2	0	100	0	55.9
<i>Interspecies hybrids</i>	100	100	66.7	95.8	4.3	100



A



B

5 Biomarkers Peak Matrix - PCA applied. Distance: Euclidean. Metric: Average

