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1 **Molecular epidemiology of Colombian *Histoplasma capsulatum* isolates shows their**
2 **polyphyletic behavior and point out raw chicken manure as one of the infections**
3 **sources.**
4
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20
21 Running Head: Molecular epidemiology of Colombian *H. capsulatum*
22

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24

25 **Key words**

26 Histoplasmosis, organic fertilizers and soil amendments, MLST technique, Hc100 nested PCR.

27

28 **Abstract**

29 The thermally dimorphic fungus *Histoplasma capsulatum* is the causative agent of
30 histoplasmosis, which is the most prevalent endemic mycosis in America. The replacement of
31 organic matter in agro-ecosystems is necessary in the tropics, and the use of organic fertilizers
32 has increased. Cases and outbreaks due to the presence of the fungus in these components
33 have been reported. In Colombia, chicken manure is the most common raw material in the
34 organic fertilizers production. In this work, we reached the isolation of the fungus from a
35 chicken manure. Then, we were able to compare genetically 3 environmental isolates with 42
36 Colombian human clinical isolates. The genetic comparison showed the environmental
37 isolates grouping together with the clinical isolates. This result suggests chicken manure as
38 one of the infection source with *H. capsulatum*. Also, the phylogenetic analysis using another
39 *H. capsulatum* isolates from databases showed that the Colombian isolates widely distributed
40 in the relation tree. This result pointed out the great genetic diversity among *H. capsulatum*
41 Colombian population.

42

43 **Introduction**

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44 Histoplasmosis is a disease caused by the thermally dimorphic fungus *Histoplasma*
45 *capsulatum (H. capsulatum)* that has been documented in all continents except Antarctica. In
46 America, it is highly endemic, particularly in the Ohio and Mississippi river valleys. Estimates
47 based on intradermal skin tests indicate that approximately 90% of the people has been in
48 contact with the fungus (1–4).

49 *H. capsulatum* infection occurs when a contaminated area is disturbed, which causes
50 aerosolization and subsequent inhalation of infective hyphal fragments and microconidia.
51 Once these particles are inhaled and reach alveoli, the body temperature stimulates a switch
52 from mycelium to yeast form. Histoplasmosis disease development depends on host factors
53 such as immune system and lung conditions as well as fungal factors such as virulence and the
54 amount of inhaled infecting particles. This interaction gives rise to different clinical forms of
55 the disease, ranging from an asymptomatic form to a severe disease that can be lethal (1–4).

56 *H. capsulatum* was first isolated from soil in 1945 by Emmons (5), who after observed
57 an environmental association between the fungus and some animals such as bats and hens (6,
58 7). Later, other researchers, through the study of outbreaks, identified sources of infection and
59 validated the relation between *H. capsulatum* with bats and birds manure (8–14). These
60 studies made it possible to delineate populations at risk of histoplasmosis, since most cases
61 have been described in ecotourists, speleologists, archaeologists, construction workers,
62 poultry farmers, chicken manure collectors, organic fertilizers producers, farmers, and in
63 general, all whose handle organic fertilizers. For the above, histoplasmosis has been defined
64 as an occupational and recreational disease (12, 15–24).

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65 In Colombia, Histoplasmosis report is not mandatory, nevertheless outbreaks and
66 cases has been described. Some of the outbreaks have been related with the handle of organic
67 fertilizers and its raw materials. Ordoñez et al. in 1997, made the compilation of 12 outbreaks
68 occurred in the Colombian Andean Region, in 10 of those outbreaks the infection source was
69 identified and in 2 the infection source were the handle of chicken manure contaminated with
70 *H. capsulatum*. Then in 2002, Jimenez et al. described an outbreak comprised a family who get
71 infected after fertilized a plant with a soil enriched with a *H. capsulatum* contaminated chicken
72 manure . In Colombia, a total of 18 outbreaks of Histoplasmosis has been described, on those
73 415 people get exposed and 188 (45.3%) were identified as infected. The source of infection
74 were mostly related with environmental sources especially caves visiting and chicken manure
75 handling (16, 20).

76 In the tropical agro-ecosystems the reconstitution of the organic matter is needed, then
77 organic fertilizers are widely used. In Colombia, the most common raw material in the
78 production of organic fertilizers and amendments is chicken manure. Therefore, if *H.*
79 *capsulatum* is found associated with chicken manure and also this is the principal raw
80 material for organic fertilizers production in Colombia, then there are a lot of people exposed
81 to the fungus.

82 In order to call the attention about the high frequency of *H.capsulatum* in tropical
83 environments and make mandatory the histoplasmosis report and to design preventive
84 measures addressed to protect the people who are mainly exposed like chicken manure
85 collectors, organic fertilizers producers, or any people who handle these products, two steps
86 are needed. First, we must to demonstrate the presence of the fungus in those substrates and

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87 second, we must to prove the relation between the isolates obtained from organic fertilizers
88 or chicken manure with the isolates obtained from human clinical cases. In a previous work,
89 we developed and applied a protocol based on the Hc100 nested PCR to search the genetic
90 material of *H. capsulatum* in composted organic fertilizers, soils samples from caves and bird
91 excretes. Then we detected *H. capsulatum* DNA in 10% of the tested samples (25). The present
92 work was addressed to isolate *H. capsulatum* from Colombian environmental samples that
93 tested positive by Hc100 nested PCR, in order to compare environmental isolates of the
94 fungus with the Colombian clinical ones based on genetic differences by Multi-Locus
95 Sequencing Typing (MLST).

96 Since the 1980's, numerous studies have evaluated the genetic variation of *H.*
97 *capsulatum* population using typing techniques such as Restriction Fragment Long
98 Polymorphism (RFLP) (26–30), Variable Number of Tandem Repeats (VNTR) (31), MultiLocus
99 Sequence Typing (MLST) (32) and Whole Genome Sequencing (WGS) (33). These studies have
100 consistently shown a strong association between phylogenetic clusters and geographic origin.
101 The currently accepted taxonomy indicates there are 8 distinct groups of *H. capsulatum*, based
102 on MLST analysis of over 130 isolates with focus on four single copy genes including fatty acid
103 desaturase (ole), β tubulin 1 (tub), ATP ribosylation factor (arf) and H antigen (H -anti) (34,
104 35). However, as with many microorganisms, taxonomic status remains dynamic with even
105 more recent research arguing *H. capsulatum* should be broken up into 4 completely separate
106 species: *H. mississippiense*, *H. ohiense*, *H. suramericanum* and *H. capsulatum sensu stricto*
107 (Sepulveda et al. 2017).

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108 The *H. capsulatum* Colombian population has been represented in previous typing
109 studies by 16 Colombian clinical isolates. Interestingly, these isolates have shown marked
110 differences among them relative to other *H. capsulatum* groups. The works by Kasuga et al.
111 (1999, 2003), and Teixeira et al. (2016) show a high degree of variability among the
112 Colombian isolates by MLST. The Kasuga's work placed the Colombian isolates in Latin
113 American clades (LAm) LAm A, LAm B and 2 solitary lineages. Similarly, the work achieved by
114 Teixeira et al. (2016) placed the Colombian isolates in the phylogenetic species LAm B1, LAm
115 A1 and LAm A2. Finally, Sepulveda et al. (2017), evaluated two additional Colombian clinical
116 isolates, MV3 and MZ5, which they argue are so divergent that they should be reclassified into
117 two different species: *H. capsulatum sensu stricto* and *H. suramericanum*, respectively. It is
118 important to point out these two isolates are different from the 16 previously used in other
119 molecular epidemiology or speciation works. Despite the fact that only a small number of
120 isolates have been studied, the existing data indicate a great diversity among the *H.*
121 *capsulatum* Colombian population (31–35).

122 Here we showed the presence of *H. capsulatum* in the Colombian environment and
123 describe the genetic analysis of *H. capsulatum* Colombian population by MLST using both
124 clinically relevant molecular markers as well as the sequences of genes used for diagnosis
125 purposes including the partial sequence of 100 KDa protein (Hc100), M antigen (AgM) and
126 two Sequences Characteristic Amplified Randomly (SCAR) (34–39).

127

128 **Materials and Methods**

129

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130 **Search for *H. capsulatum* Colombian isolates from environmental sources**

131

132 ***Environmental samples collection***

133 Samples were collected from organic fertilizers, which were the compost products
134 from an organic matter source (i.e., food scraps, pruning material, straw, or sawdust) and a
135 nitrogen source (i.e., excrement from animals, primarily poultry or other birds), soil from cave
136 floors and bird and bat droppings. Between 500 and 1000 grams of each environmental
137 sample was placed in a plastic zip lock bag and labeled with the date, geographical
138 coordinates, and the type of sample (raw material, composted material, cave soil, bat excretes
139 or chicken excretes). Samples were then sent to the Grupo de Micología Médica, School of
140 Medicine, Universidad de Antioquia to be processed.

141 393 samples were collected between 2010 and 2017. Of these 393 samples, 273
142 (69.5%) were composted fertilizers and organic amendments. A total of 120 (30.5%) samples
143 did not have composting treatment. In the last group, 21 (5.3%) samples were from caves
144 floors and/or bat droppings and a total of 99 (25.2%) samples were from bird depositions or
145 chicken manure.

146

147 ***DNA extraction from environmental samples using the "FastDNA SPIN Kit for Soil"***

148 The FastDNA SPIN Kit For Soil® (MP Biomedicals, Santa Ana, CA, USA) was used to
149 extract DNA from environmental samples using the manufacturer's instructions with some
150 modifications. Briefly, the extraction was performed using the supernatant obtained from the
151 suspension of 10 g of sample in 30 ml of saline solution containing 0.001% Tween 80 and

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152 0.1% antibiotics (gentamycin and penicillin). The suspension was stirred vigorously for 1 min
153 and allowed to settle for 20 min. This procedure was repeated twice. After the last stirring, the
154 suspension was allowed to settle only until the largest particles were settled; then, 300 µl of
155 supernatant was collected for DNA extraction. The other modification consisted of an
156 increased contact time between the sample and kit reagents (25).
157

158 ***Nested Hc100-PCR assay for searching H. capsulatum in environmental samples***

159 Two sets of specific primers targeting a fragment of the *Hcp*100 gene were used (36,
160 37). The conditions of the assay for organic fertilizers were described by Gomez et al. (2018)
161 (25). In the first reaction, 10µl of DNA was added to 50µl total reaction mix with a final
162 concentration of 10 mM Tris - HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 1U Taq polymerase
163 (Thermo Scientific, Ref: EP0402. Waltham, M A, USA), 0.2 mM of each primer (HcI-HcII), and
164 0.2mm deoxynucleoside triphosphate (Thermo Scientific, Ref: R0181. Waltham, M A, USA).
165 The mixture for the nested PCR was similar, except using 2µl of the product of the first PCR
166 and 0.2mM of the inner primers (HcIII-HcIV). Temperatures and times for the first reaction,
167 containing external primers HcI-HcII were one cycle at 94°C for 5 min; 35 cycles at 94°C for
168 30s, 66°C for 1 min, and 72°C for 1 min, and then a final cycle of 72°C for 5 min. For the second
169 step, the reaction consisted of a cycle at 94°C for 5 min; 35 cycles at 94°C for 30s, 65°C for 30s,
170 and 72°C for 1 min, and then a final extension cycle at 72°C for 5 min. Synthesis of the primers
171 was performed by Integrated DNA Technologies (IDT, Coralville, IA, USA).
172

173 ***Agarose gel electrophoresis***

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174 Agarose gels (Amresco, Ref.: N605-500G, Solon, OH, USA) prepared at 1.5% in Tris-
175 borate EDTA buffer (TBE) were used to visualize the amplification products of the Hc100
176 nested PCR. Electrophoresis was performed for 40 min at 80 V with 10 µl of the PCR product
177 and 5 µl of GelRed Nucleic Acid Gel stain (Biotum. Ref.: 41003, Hayward, CA, USA) in each lane.
178 The bands were visualized and documented in a UV transilluminator (Doc Gel ™, BioRad.
179 Hercules, CA, USA).

180

181 ***H. capsulatum* recovery in microbiological cultures from environmental samples that**
182 **tested positive in the Hc100 nested PCR**

183 The culture was performed using the supernatant obtained from the suspension of 10
184 g of sample in 30 ml of sterile saline solution 0.85% containing 0.001% Tween 80 (Sigma, ref.
185 P4780. St. Louis, MO, USA), 100 pg/ml oxytetracycline (MK. Cali, Valle, COL), and 0.1%
186 antibiotics (gentamycin and penicillin (MK. Cali, Valle, COL)). The suspension was stirred
187 vigorously for 1 min and allowed to settle for 20 min. This procedure was repeated twice.
188 After the last stirring, the suspension was allowed to sit until the largest particles were
189 settled; then, the supernatant was collected and serial dilutions were performed 1:10, 1:100
190 and 1:1000, from each dilution were plated 200 µl in Mycosel agar (BBL™; ref. 211462.
191 Franklin Lakes, NJ, USA) by duplicate. Cultures were incubated at room temperature for 2
192 months and plates were visually inspected on days 5, 10, 15, 30, 45 and 60 in order to look for
193 growth of colonies with morphology that resemble *H. capsulatum*.

194 The colonies resembling *H. capsulatum* were identified as cottony colonies, raised,
195 hard- edged, white, cream or light coffee and slow growth. Each candidate colony was

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196 subcultured on a fresh Mycosel agar and the microscopic examination was done with
197 Lactophenol blue to observe the characteristics indicative of *H. capsulatum* such as thin
198 septate hyaline hyphae, thin wall microconidia and tuberous macroconidia. Colonies with
199 these features were transformed into the yeast form using the procedure described in Gomez
200 et al. (2018) (25). Isolate identities were confirmed with sequencing of *hcp*100 gene.
201
202 ***H. capsulatum* recovery in mouse model from environmental samples that tested positive**
203 **in the Hc100 nested PCR**
204
205 ***Assays conducive to stablish the sensitivity of the mouse model***
206 The efficacy of isolating *H. capsulatum* from the environmental sample was evaluated
207 with organic fertilizers samples contaminated with 3000 CFU/ml in mycelial stage (Gomez et
208 al. in preparation manuscript). 1:10, 1:100 and 1:1000 dilutions were prepared from the
209 sample supernatant and from each dilution, 2 mice were inoculated in the peritoneum cavity
210 with 500 µl. The animals maintenance is explained below.
211
212 ***Environmental samples preparation for mice inoculation***
213 Hc100 nested PCR positive samples were prepared by suspending 10 g of sample in
214 100 ml of saline solution containing 0.1% antibiotics (gentamycin and penicillin). The
215 suspension was stirred vigorously for 1 minute and allowed to settle for 20 minutes. This
216 procedure was repeated twice. After the last stirring, the suspension was allowed to settle 40
217 minutes; then, the supernatant was collected for mice inoculation.

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218

219 ***Animals' maintenance and inoculation***

220 Specific pathogen-free male Balb/C mice (6–8 week old, 18–22 g weight) were
221 obtained from the Laboratory Animal Center at the Corporación para Investigaciones
222 Biológicas (CIB, Medellin, Colombia). Mice were housed in a caging system (RAIR HD Super
223 Mouse 750TM Racks system, Lab Products, Inc. Seaford, DE, USA) equipped with high
224 efficiency particulate air (HEPA) filters with controlled room temperature at 20-24°C; and 12-
225 h light/dark cycles, under sterile conditions and provided with sterilized food and water *ad*
226 *libitum*.

227 Mice were inoculated in the peritoneum cavity with 500µl of the supernatant. After 17
228 days, mice were euthanized and the affected organs (spleen, liver and lungs) were extracted.
229 The organs were macerated and cultured in BHI agar (Brain Hearth Infusion Agar, BBL ™; ref.
230 211065. Franklin Lakes, NJ, USA) supplemented with 1% glucose (Sigma,Ref. G5400. St. Louis,
231 MO, USA), 0.001% L-cysteine (Sigma, ref. C - 7755. St. Louis, MO, USA) and 5% anticoagulated
232 blood, then cultures were incubated at 37°C in 5% CO_2 atmosphere for two weeks and at room
233 temperature in Mycosel agar for 2 months, in order to look for *H. capsulatum* compatible yeast
234 colonies.

235

236 ***Ethics statement.***

237 This study was performed according to recommendations of European Union,
238 Canadian Council on Animal Care, and Colombian regulations (Law 84/1989, Resolution No.

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239 8430/1993). The protocol was approved by the Ethics' Research Committee at the CIB
240 (Comité de ética, electronic consultation on July 24th, 2014).
241
242 **Collection of Colombian *H. capsulatum* clinical isolates**
243
244 ***H. capsulatum culture***
245 42 human clinical isolates of *H. capsulatum* were obtained from the CIB (CIB, Medellin,
246 Colombia). All isolates were transformed into the yeast form following the procedure were
247 described in Gomez et al. (2018) (25).
248
249 ***DNA extraction from H. capsulatum isolates in yeast phase***
250 The phenol-chloroform-isoamyl alcohol method was used for DNA extraction from the
251 *H. capsulatum* isolates in yeast phase (40). The DNA concentration and quality were evaluated
252 with a NanoDrop ND1000 spectrometer (Thermo Scientific, Wilmington, DE, USA) and 1%
253 agarose gel electrophoresis, respectively
254
255 **Molecular epidemiology comparison among *H. capsulatum* population.**
256
257 ***MLST technique***
258 The characteristics of the technique to compare the isolates of *H. capsulatum* were
259 described by Kasuga, Taylor and White (1999) (34). The primers were synthesized by

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260 Integrated DNA Technologies (IDT, Coralville, IA, USA). Table S2 describes the characteristics
261 of each primer.

262 The standardized conditions for each MLST PCR assay and primers used are described
263 on Table S2.

264

265 ***Other selected and evaluated H. capsulatum genome regions to compare isolates***

266 Given the difficulties associated with obtaining isolates from environmental samples,
267 diagnostic protocols originally designed for clinical samples were standardized and applied in
268 environmental samples to obtain sequences directly from the samples to enable comparison
269 between clinical and environmental *H. capsulatum* isolates. To fulfil this goal only the first
270 round of the Hc100 Nested PCR and antigen M nested PCR assays were done because the
271 amplification product is bigger. Additionally, two PCR assays targeting two Sequences
272 Characteristic Amplified Randomly (SCAR) in the *H. capsulatum* genome were also used. Table
273 S2 describes the characteristics of each primer and PCR reaction.

274

275 ***Sequences analyses***

276 Bidirectional sequencing of the PCR amplification products were performed using the
277 chain termination method. The method used ABI 3730XL DNA sequencing technology with
278 quality criteria QV20 (Macrogen Inc., Geumcheon-gu, Seoul, Korea). The obtained sequences
279 were edited manually based on the chromatograms. The mold and complementary sequences
280 were aligned and the consensus sequence was obtained using Geneious 11.0.2. Software.
281 BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to verify that the sequenced PCR

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282 products belonged to *H. capsulatum.* It was obtaining the information of the 80 sequences
283 from TreeBASE #1063, the matrix was separated gene by gene according to the length and the
284 order reported by the author (34). Additionally, the sequences of 85 isolates were
285 downloaded from NCBI using the access codes by the Batch entrez program. Additionally, the
286 sequences were aligned and concatenated using Geneious 11.0.2. Software, and the distance
287 trees were constructed using the Maximum Likelihood and 10000 UltrafastBootstrap methods
288 using IqTree 1.4.4. Software. The tree obtained was visualized using FigTree v.1.4.3.
289
290 **Results**
291
292 **Detection of *H. capsulatum* in environmental samples by Hc100 nested PCR**
293 Out of the 393 environmental samples, a total of 39 (9.9%) were positive by Hc100
294 nested PCR. Of these positive samples, 21 (5.3%) were composted fertilizers and organic
295 amendments, 17 samples (4.3%) were from bird depositions or poultry manure and one
296 (0.3%) sample was from a cave floor/bat droppings. Interestingly, we found high positivity
297 rates of samples without composting treatment 18/120 (15%) compared with the samples
298 that were previously composted 21/273 (7.7%).
299
300 **Recovery of *H. capsulatum* in microbiological cultures from Hc100 nested PCR positive**
301 **environmental samples**
302 The 39 positive samples by the Hc100 nested PCR were seeded into Mycosel culture
303 medium. However, only one sample taken from non-composted chicken manure produced

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304 viable *H. capsulatum* cultures. From this single sample, 3 *H. capsulatum* colonies were
305 obtained and used as different individuals for molecular analyses. Interestingly, while 2 of
306 these isolates were morphologically similar, the third was distinct and did not fully switch to
307 the yeast form. Abundant growth of bacteria and molds, such as *Penicillium* spp*, Aspergillus*
308 spp*, Trichoderma* spp and *Geotrichum* spp were also observed in the *H. capsulatum* cultures.
309

310 **Recovery of *H. capsulatum* in mouse model from Hc100 nested PCR positive**
311 **environmental samples**

312 In attempt to obtain more isolates, the 39 positive samples from the Hc100 nested PCR
313 were also used to inoculate mice. 5 to 8 mice were used to inoculate each positive sample,
314 around 9% of the mice died in the first 3 days after inoculation. Despite the use of broad-
315 spectrum antibiotics such as oxytetracycline in the sample preparation, the administration of
316 antibiotics to the mice before and after environmental samples inoculation and the rigorous
317 observation of the mice tissue cultures in Mycosel and BHI, we were not able to obtain
318 environmental isolates of *H. capsulatum*, although as it was described before *H.capsulatum*
319 was recovered from mice inoculated with environmental samples artificially contaminated
320 with 3000 CFU/ml of this fungus.
321

322 **MLST analysis of Colombian *H. capsulatum* populations**

323 The conditions of each PCR protocol were standardized (Table S2). Then the
324 sensitivity, specificity and the ability to detect *H. capsulatum* DNA from environmental
325 samples could be evaluated (Figure 1). All protocols, including both typing and diagnostic

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326 methods, were tested to detect *H. capsulatum* from environmental samples. Although some of
327 them showed a good limit of detection (Fig 1, Lanes 1-7) and specificity (Fig 1, Lanes 8-13)
328 with spiked samples with *H. capsulatum* DNA, none of these methods produced an amplicon
329 from the 39 samples shown to be positive with the Hc100 nested PCR.

330 The standardized PCR protocols were used to obtain the sequences from all 45
331 Colombian isolates (42 human clinical and 3 environmental isolates) (Table S1), after the PCR
332 products were sequenced, the consensus sequence was obtained. The length for each locus
333 was as follows: *arf* 478 bp, *anti H* 413 bp, *ole* 428 bp, *tub* 263 bp, *100kda* 209 bp, *anti M* 276
334 bp, scar220 208 bp, scar230 262 bp, ITS 605 bp. The characteristics of each gene are shown in
335 the Table S2.

336 Subsequently, the first matrix was constructed using the sequences of the genes in the
337 following order: *arf*, *antiH*, *tub1* and *ole*. The matrix included 225 sequences, had a length of
338 1582 bp and was partitioned by the type of sequence itself: CDS, exon or ITS. The evolutionary
339 model was established as K2+G4. 17 phylogenetic groups were identified with similar
340 distribution as reported by Kasuga, 2003 and Teixeira, 2016 (Figure 2).

341 Additionally, three more matrices were built that only included the Colombian isolates,
342 given that only from those were obtained the sequences corresponding to Hcp100 gene, M
343 antigen gene, SCAR 220, SCAR 230 and the ITS region. In this analysis, the first matrix
344 included *arf*, *antiH*, *tub1* and *ole*. The second matrix included the sequences obtained using the
345 PCR products of the diagnosis genes target, those sequences were aligned in the order as
346 shown above and a third matrix included all nine sequences. In all matrices, the
347 environmental isolates were grouped with the clinical isolates. When the 3 different matrices

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348 were analyzed, the trees had the same topology, this suggest that the classical genes are
349 adequate to achieve the genetic comparison inside the *H. capsulatum* population (Figure 3).
350 According to the genes characteristics for MLST, the diagnostic genes have less number of
351 informative sites per gene than the classical targets (Table 1).
352
353 **Discussion**
354 Histoplasmosis is the most prevalent endemic mycoses in America. *H. capsulatum*, the
355 causative agent, is a thermally dimorphic fungus associated with bat/cave environments, bird
356 droppings, and other diverse environmental sources such as soil and compost.
357 People exposed to *H. capsulatum* can be either asymptomatic or experience life
358 threatening histoplasmosis depending on a combination of risk factors (4, 41). A better
359 understanding of the genetic diversity of environmental and clinical isolates *H. capsulatum* in
360 context of geographical location can help us better understand the risk factors for developing
361 histoplasmosis. In this study, we showed how *H. capsulatum* strains in Colombia show a
362 particularly high degree of variation compared to other geographic regions through the
363 phylogenetic analysis of over 3 environmental and 42 human clinical isolates.
364 Using Hc100 nested PCR, we were able to detect *H. capsulatum* in 39 (9.9%) of 393 of
365 environmental samples studied. The fact that of the positive samples, 21.9% were found in
366 non-composted samples compared to only 7.7% of composted samples, suggests that a well-
367 done composting process could reduce the risk of exposure to *H. capsulatum* when
368 manipulating the organic fertilizer final product. This is because composting process is a
369 spontaneous decomposition of organic matter, mainly aerobic, in which bacteria and fungi

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370 mainly participate. The organic matter is transformed in a free of toxins and pathogenic
371 microorganisms fertilizer due to the action of the saprophyte microorganisms. First, they are
372 better competitors for space and nutrients and second due to their metabolic activity, which
373 generates an increase in the temperature. These two actions reduce the pathogenic
374 microorganisms population (42–44). However, the composting process at the beginning,
375 continuing being a high risk infection source, since we achieve the isolation of *H. capsulatum*
376 from a raw/not composted chicken manure, that happens to be the most used raw material in
377 organic fertilizers in Colombia.

378 For the isolation of *H. capsulatum* from environmental samples, the mouse model has
379 been considered the golden standard technique, but, in this study, we could not isolate the
380 fungus using it. This procedure is time-consuming, expensive, requires training to handle the
381 mice and based on previous studies it has a low success rates between 0-50% (5, 6, 8, 19, 20,
382 45–51). Furthermore, we found the high microbial background in our environmental samples
383 led to sepsis and subsequent death in many of the mice, further emphasizing the high cost of
384 such experiments that ultimately failed to recover the *H.capsulatum* isolates. Moreover, the
385 search for *H. capsulatum* using the mouse model requires the fungus to overcome the
386 saprophytic microorganisms, evade the immune system and establish an infection in the
387 mammal host before it can be isolated from affected tissues (5, 6, 8, 19, 20, 45–51). One
388 challenge associated with this method is that if the fungus has been in the environment for
389 long time without the exposure to a mammal host, the genes associated with pathogenicity
390 may not be activated. For example, when the α-(1-3)-glucan cell wall content has been studied
391 in *H. capsulatum*, the fungus has been separated into chemotypes I and II; this classification

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392 has been related also with its virulence. Most *H. capsulatum* isolates described are chemotype
393 II, but can lose virulence spontaneously by successive passaging in culture by loss of α-(1-3)-
394 glucan in their cell wall (52–54). Nowadays, it is possible to compare isolates using the
395 proteome analysis. This approach could be used to elucidate genes related to pathogenicity by
396 comparing gene activity in environmental and clinical isolates (55–57). If environmental
397 isolates have not activated the virulence genes like the clinical isolates, then the use of the
398 mouse model for the isolation of *H. capsulatum* from environmental samples would need to be
399 reconsidered to be the gold standard technique .

400 In contrast, we were able to isolate *H. capsulatum* by the direct culture of the
401 environmental samples. In cases with high microbial background such as chicken manure
402 samples, we were able to limit contamination by adding antibiotics to the culture media. Even
403 though this method also had a low success rate, the recovery of just a few isolates proved to
404 be very valuable for our study. From our analysis of the 3 isolates from the non-composted
405 chicken manure sample, we were able demonstrate viable fungus was present in the raw
406 materials. This has implications for worker safety in industrial contexts, and its necessary take
407 actions regarding protective measures like wearing high efficient respirators, gloves, boots,
408 long sleeve shirts and showering at the end of the day. Following these few recommendations
409 is the best way to avoid the occurrence of occupational outbreaks of histoplasmosis (58). In
410 order to promote the knowledge about histoplasmosis and how to avoid the infection with *H.*
411 *capsulatum*, we wrote a booklet to teach people about the disease, the signs and symptoms,
412 whom is usually expose and how to protect themselves (59).

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413 Given the difficulties associated with obtaining isolates from environmental samples,
414 we probed diagnostic protocols probed, to obtain sequences directly from the environmental
415 samples to enable comparison between clinical and environmental *H. capsulatum* isolates.
416 However, none of these methods produced an amplicon from the 39 samples shown to be
417 positive with the Hc100 nested PCR. Also, given that the trees obtained with the 3 different
418 had the same topology, the classical genes proposed by Kasuga et al. are adequate to achieve
419 the genetic comparison inside the *H. capsulatum* population (34, 35, 37–39, 60).

420 Then, Our successful recovery of three *H. capsulatum* isolates from the chicken manure
421 sample enabled us to genetically compare these strains to 42 human clinical isolates from
422 Colombia, as well as other isolates from around the world by the classical MLST (34, 35). Our
423 MLST analysis revealed that the isolates from chicken manure are most closely related to the
424 clinical strains in Colombia. This finding strongly implicates chicken manure as a source for
425 exposure and infection by *H. capsulatum*. Previous studies of histoplasmosis outbreaks have
426 used similar approaches and also been able to establish associations between environmental
427 and clinical *H. capsulatum* isolates (26, 32, 35).

428 Our work confirming the relatively high diversity of Colombian isolates compared to
429 populations in other geographical regions (32, 33, 35). Although it is not yet certain why there
430 is such a high diversity, it is likely related to Colombia's ecological and geographical
431 characteristics. Colombia has a unique location that serves a crossing point for migratory
432 birds and bats, which can carry and seed the fungus. This mechanism of dispersal has been
433 demonstrated by studies of the bat *Tadarida brasiliensis*, whose range correlates well with the
434 epidemiological map of histoplasmosis. This mechanism may explain the presence of

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435 Colombian isolates in the clades that traditionally include isolates from other countries the
436 bat visits in its migratory route like the United States and Brazil. Furthermore, *H. capsulatum*
437 has also been isolated from tissues and excretes from both birds and bats (51, 61–66). Also,
438 since tropical soils tend to be nutritionally poor, organic fertilizers made on chicken manure
439 sources are commonly used, which creates increased habitat for *H. capsulatum* that overlaps
440 with human activity. Our data implicates a mechanism of clinical infection originating from
441 non-composted chicken manure sources. It is therefore likely that the risk of histoplasmosis
442 can be reduced through complete composting of chicken manure before extensive handling by
443 people. Risk may also be reduced by, the use of personal protection when exposed to risky
444 activities like the visit to caves, the demolition or cleaning of old buildings or the manipulation
445 of excreta of birds and bats and the inclusion of the *H. capsulatum* search during the
446 composting process. Following these recommendations, cases and outbreaks could be
447 prevented.
448

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651 **Data Accessibility**

652 Genbank accession numbers ADP ribosylation factor (arf) MH338036 to MH338080, H
653 antigen precursor (*anti-H*) MH122839 to MH122883, Delta-9 fatty acid desaturase (*ole1*)
654 MH338126 to MH338170, Alpha-tubulin (*tub1*) MH338081 to MH338125, Hcp100 gene, 100
655 kDa protein MH122794 to MH122838, internal transcribed spacer (ITS) MH339542 to
656 MH339586, SCAR markers 220 and 230 MH348521 to MH348610.
657

Table 1. List the sequence name, the length in the present analysis and the number and percentage of the informative sites.

Sequence	Length	Informative sites	Percentage informative sites
arf	435	7	1,6
anti H	373	6	1,6
ole	383	3	0,7
tub	218	3	1,3
ITS	608	4	0,6
Hcp 100	184	1	0,5
anti M	276	7	2,5
SCARS 220	180	5	2,7
SCARS 230	152	1	0,6

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Table 1

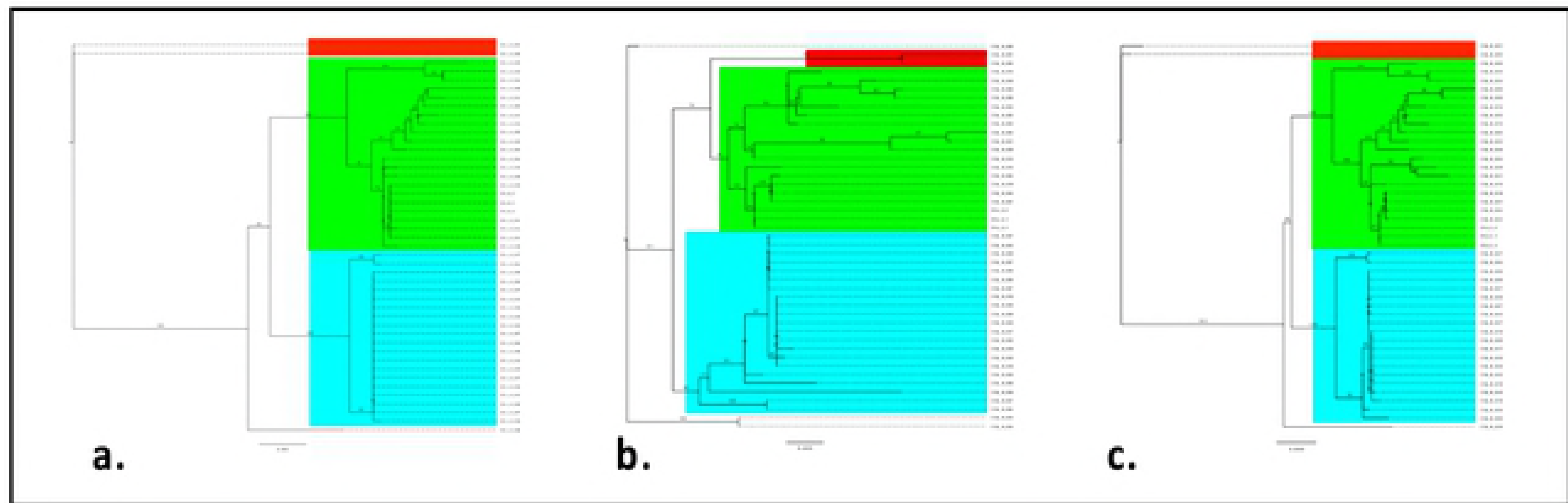

Figure 3. The phylogenetic analysis of the *H.capsulatum* Colombian population. All trees showed the same topology although there were built with different group of genes in the order as follow, **a.** *arf*, *antiH*, *tub1* and *ole.* **b.** Hcp100 gene, M antigen gene, SCAR 220, SCAR 230 and the ITS region and **c.** Consensus tree of all 9 sequences *arf*, *antiH*, *tub1*, *ole,* Hcp100 gene, M antigen gene, SCAR 220, SCAR 230 and the ITS region.

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Figure 3

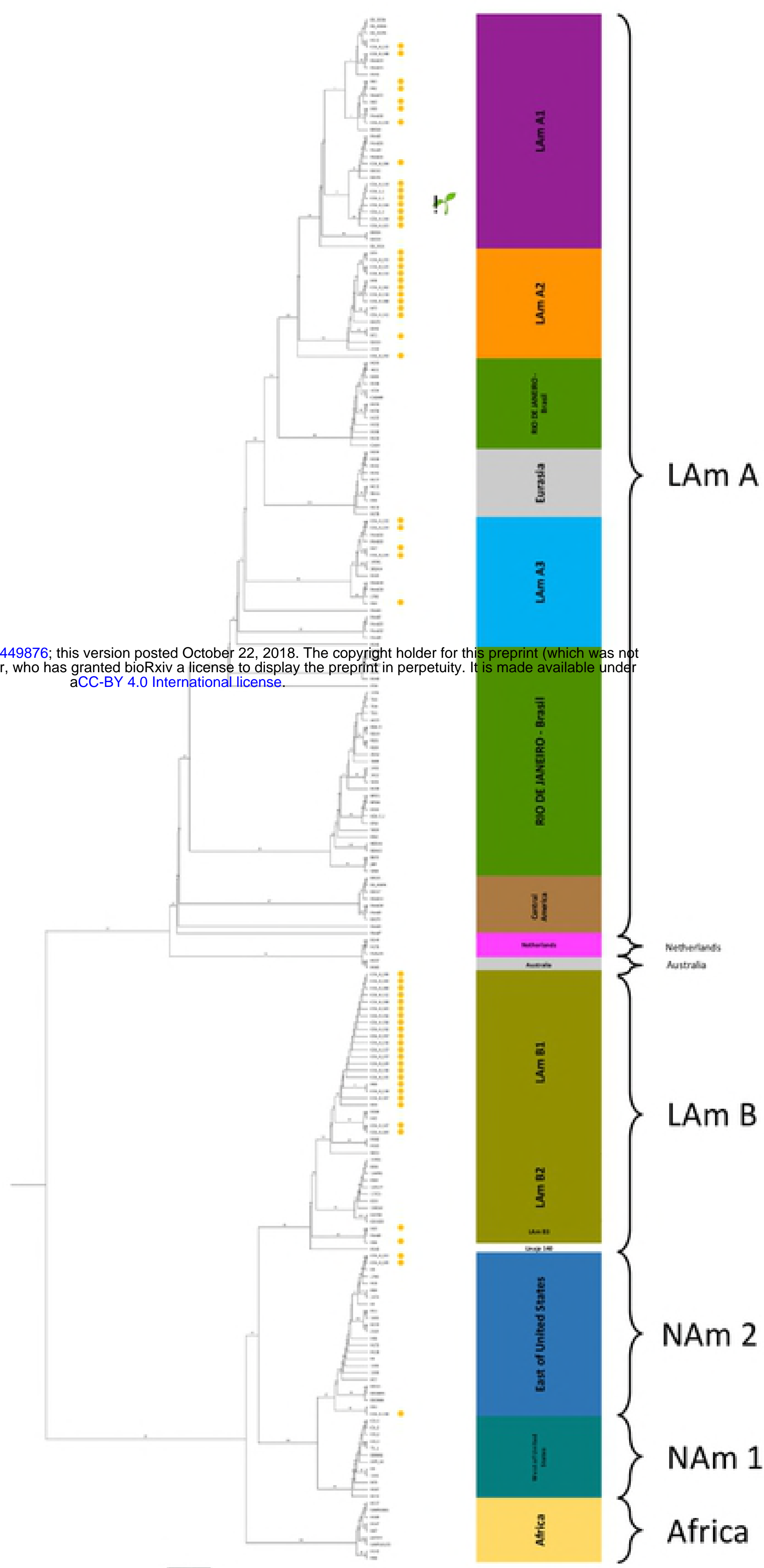

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Figure 2. Genetic comparison of *H.capsulatum* Colombian isolates. The groups obtained, were separated when bootstraps were over 70%. The contrast between our results and the Teixeira et al. (2016) (middle list) and the Kasuga et

Figure 2

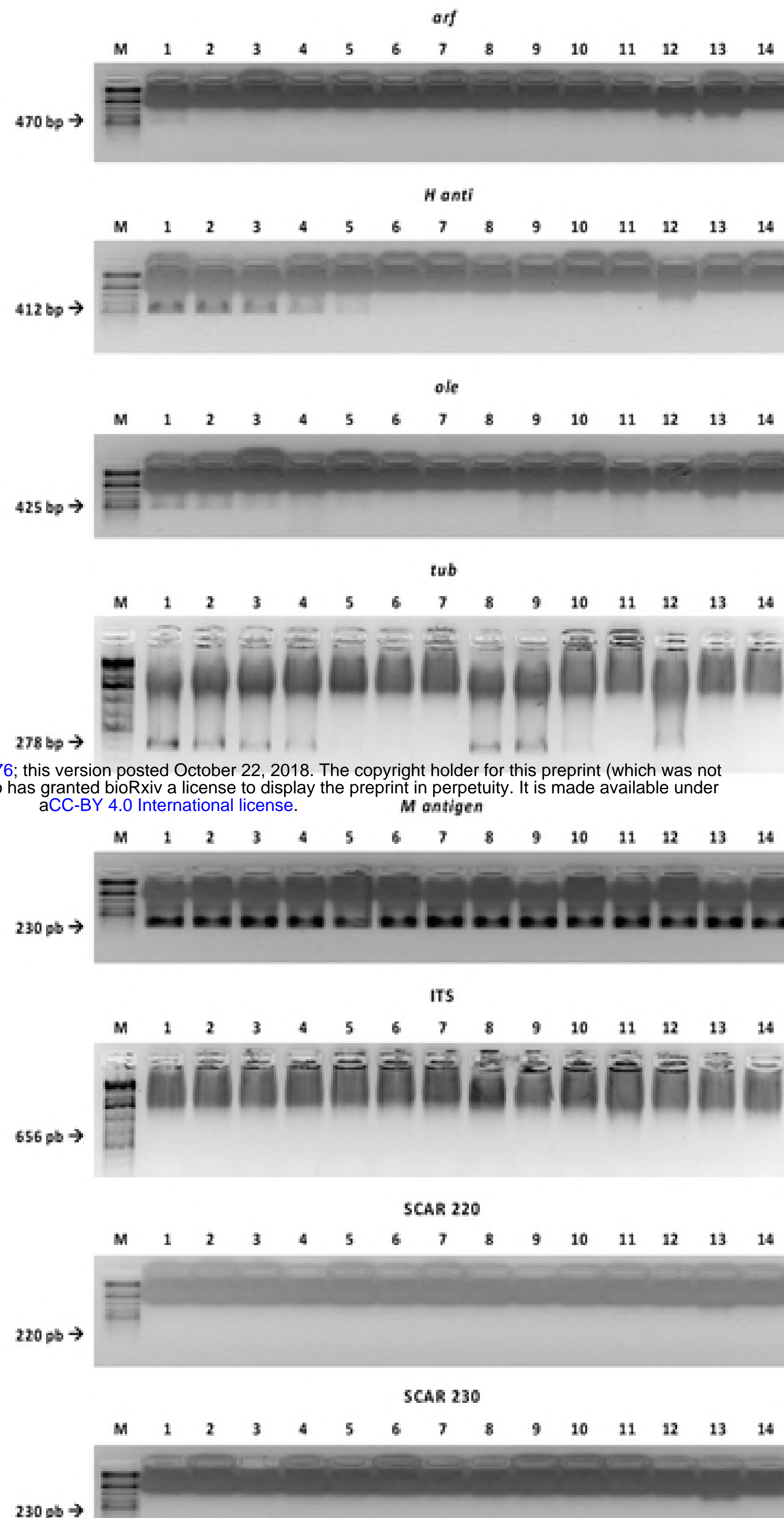

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Figure 1. Detection limit, specificity and the ability to detect *H. capsulatum* DNA in environmental samples with the MLST and diagnostic PCR reactions. Line M: marker, line 1: 2 ng/µl, line 2: 1 ng/µl, line 3: 500 pg/µl, line 4: 200 pg/µl, line 5: 100 pg/µl, line 6: 50 pg/µl, line 7: 20 pg/µl, line 8: *Paracoccidioides brasiliensis* sensu stricto (Pb 18), line 9: *Paracoccidioides* spp (Pb 339), line 10: *Paracoccidioides restrepiensis* (PB 60855), line 11: *Coccidioides* spp, line 12:

Figure 1