Fungi with history: Unveiling the mycobiota of historic documents of Costa Rica

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Abstract

Through nondestructive techniques, we studied the physicochemical characteristics and mycobiota of five key historic documents from Costa Rica, including the Independence Act of Costa Rica from 1821. We determined that for documents dated between 1500 and 1900 (i.e., the Cloudy Days Act, the Independence Act, and two documents from the Guatemalan Series from 1539 and 1549), the paper composition was cotton, whereas the 1991 replicate of the Political Constitution from 1949 was made of wood cellulose with an increased lignin content. We also determined that the ink employed in 1821 documents is ferrogallic, i.e., formed by iron sulfate salts in combination with gallic and tannic acids. In total, 22 fungal isolates were obtained: 15 from the wood-cellulose-based Political Constitution and seven from the other three cotton-based documents. These results suggest that cotton-based paper is the most resistant to microbial colonization. Molecular identifications using three DNA markers (i.e., ITS nrDNA, beta-tubulin, and translation elongation factor 1-alpha) classified the isolates in eight orders and ten genera. The most frequent genera were *Cladosporium*, *Penicillium*, and *Purpureocillium*. Of the isolates, 95% presented cellulolytic activity correlated to their ability to cause deterioration of the paper. This work increases the knowledge of the fungal diversity that inhabits historic documents and its relationship with paper composition and provides valuable information to develop strategies to conserve and restore these invaluable documents.

Keywords

Biodeterioration; historic documents of Costa Rica, Independence Act of Costa Rica, *Cladosporium*, *Penicillium*, *Purpureocillium*, cellulolytic activity.

Introduction

Because biodeterioration can lead to the damage of historic documents, artwork, monuments, or buildings, its study is fundamental for the conservation of cultural heritage (Sterflinger and Pinar 2012; Palla and Barresi 2017; Vieto *et al.* 2021; Ranalli and Zanardini 2021; Ranalli *et al*. 2005). The prevention of biodeterioration and development of adequate conservation and restoration strategies cannot be an unscripted process; it is necessary to undertake diagnoses of these valuable pieces of our history and art, which include chemical characterization and the study of microbial diversity together with the physiological characteristics of biodeteriogens (Palla and Barresi 2017; Negi and Sarethy 2019).

Valuable cultural and historic objects, such as relevant paintings, ancient sculptures, and historic documents, can be seen as substrates on which microorganisms can thrive and cause damage. Specifically, paper-based documents contain biodegradable organic constituents that fungi can use as a substrate (Jia *et al.* 2020; Pyzik *et al.* 2021). The term "paper" is a general concept that encompasses all thinly laminated material that is produced with vegetable fiber pulp or other materials ground and mixed with water, dried, and hardened. Historically, vegetable fibers have been extracted from natural sources, such as straw, silk, hemp, flax, cotton, and the bark of different trees, among others. The content of cellulose and other components of paper can vary depending on its origin, generating papers that are more or less resistant to biodegradation as a consequence. For example, it is well known that cellulose fibers have high purity in cotton and linen papers, which results in papers with greater durability and resistance to biodeterioration (Negulescu *et al.* 1998; Daria *et al.* 2020).

69 Damage produced by fungi that is normally present on paper —including staining,
70 material weakening, and partial or complete destruction of documents— can occur in the long term
71 (Sequeira *et al.* 2019). Besides the alterations caused to the documents, the health of curators and
72 people involved in archives or museums can also be threatened if the spore production is elevated
73 or mycotoxins are produced (Sterflinger and Pinzari 2012). The study of fungi responsible for the
74 biodegradation of paper began in 1818 with the pioneering work of Christian Gottfried Ehrenberg
75 (Sterflinger and Pinzari 2012). To date, diverse fungi have been identified in old paperwork, such
76 as *Aspergillus*, *Chaetomium*, *Cladosporium*, *Penicillium,* and *Trichoderma*; occasionally, new
77 species can even be found (Coronado-Ruiz *et al.* 2018; Sequeira *et al.* 2019). Mesquita *et al.* (2009)
78 isolated, identified, and characterized the microbiota from historic documents dated between
79 1860–1939 in the archive of the University of Coimbra and found fourteen fungal genera, of which
80 *Aspergillus*, *Cladosporium*, and *Penicillium* were the most common. Our research group recently
81 isolated nineteen fungi from a nineteenth-century French collection of drawings and lithographs
82 in the custody of Universidad de Costa Rica (Coronado-Ruíz *et al.* 2018). The fungi were
83 molecularly identified as *Arthrinium, Aspergillus*, *Chaetomium*, *Cladosporium*, *Colletotrichum*,
84 *Penicillium*, and *Trichoderma*; a great majority of them showed cellulolytic activity. Many fungal
85 species found in historic paper-based documents contain enzymatic activity related to
86 biodeterioration, which allows fungi to use these surfaces as a source of carbon (Coronado-Ruíz
87 *et al.* 2018; Pinheiro *et al.* 2019; Vieto *et al.* 2022). The enzymatic machinery to take advantage
88 of paper as a source of carbon has been reported in fungi isolated from historic documents and
89 includes the presence of exoenzymes with cellulase activity (Puškárová *et al.* 2019; El Begardi *et*
90 *al.* 2014), lignocellulolytic (Mazzoli *et al.* 2018) glucanase, and laccase (Sterflinger and Pinzari
91 2012).

The National Archive of Costa Rica (NACR) —called Archivos Nacionales (National Archives) before 1948— is where the most treasured documents in Costa Rica are preserved; these include the Cloudy Days Act (Acta de los Nublados, September 28, 1821), in which authorities of the Municipality of León, in the Captaincy General of Guatemala, expressed their position on Central American Independence; the Political Constitution with all historic changes, including the abolition of the Costa Rican army (Jaén García 2019; Chacón León 2021); and perhaps the most important historic document in the country: the Costa Rican Independence Act (Acta de Independencia, October 29, 1821). These invaluable documents are in addition to more than 20,000 linear meters of other paper-based documents that contain the history of Costa Rica and that are in the custody of NACR. Due to the tropical peculiarities of the country —such as high humidity, heat, long rainy seasons, and sometimes inadequate storage conditions— documents and artworks are constantly threatened and come under continuous biodeterioration, making the conservation of the country's cultural heritage a challenge (Silva, 2011).

The aim of this work was to characterize the paper composition and evaluate the presence and cellulolytic activity of culturable fungi in historic documents from the National Archive of Costa Rica, including invaluable documents such as the Independence Act of Costa Rica. This information enables restorers to establish guidelines for the preservation and restoration of paper-based historic documents.

Materials and Methods

Sampling of documents

115 Permits to sample the historic documents were obtained from the Institutional Commission
116 of Biodiversity of the University of Costa Rica (resolution Nº 186) and authorities of the NACR.
117 Historic documents stored at NACR were sampled between March and September 2019. The
118 documents were: (i) Political Constitution redacted in 1949 (1991 replicate), (ii) Cloudy Days Act
119 from 1821 (Acta de los Nublados), (iii) Independence Act of Costa Rica from 1821, and (iv) two
120 documents from the Guatemalan Series from 1539 and 1549 (Fig. 1). For fungal isolation, careful
121 rubbing with sterile cotton swabs over the surface of the documents was performed, especially
122 seeking signs of biodeterioration such as dark spots. The swabs were then saved inside Falcon
123 tubes for transport to the laboratory.
124
125 *Material characterization by attenuated-total-reflection Fourier-transform infrared spectra (ATR-*
126 *FTIR).*
127
128 ATR-FTIR was used to determine functional groups and distinguish cellulosic materials.
129 These ATR-FTIR spectra were recorded using a portable spectrophotometer (Bruker Alpha II,
130 Canada) with platinum ATR mode and monolithic diamond crystal. The spectral resolution was 4
131 cm^{-1}, in wavenumber range 400–4,000 cm^{-1} with 99 scans. To carry out the identification, the
132 "Database of ATR-FT-IR spectra of various materials" (Vahur *et al.* 2016) was used.
133
134 *Material characterization by X-ray fluorescence (XRF).*
135
136 X-ray fluorescence (XRF) was used to determine the elemental composition of the
137 material, especially the presence of metallic ions, such as iron and calcium, among others. This

138 technique is especially important in the characterization of inks. The XRF spectra were recorded
139 with a portable XRF spectrophotometer (Elio, XGLab; Bruker, Italy) measured at the Kα line of
140 manganese (resolution 140 eV), a SDD detector (active area 25 mm^2, fluorescence angle 63°,
141 incident angle 90°) and a distance of 14 mm from the detector to the sample. The electric current
142 was adjusted to 80 μA, with a voltage of 50 kV and a measuring duration of 300 s. Software XRS-
143 FP2 (CrossRoads Scientific, USA) was used for data analysis, maintaining a noise signal of 0.5.
144
145 *Fungal cultivation strategy*
146
147 Samples were processed in the laboratory 2-4 hours after sampling. Cotton swabs were
148 immersed into sterile phosphate-buffered saline solution (PBS; 400 μL, 1X; Thermo Fisher
149 Scientific, USA) and homogenized using a vortex (40 s). Each sample (100 μL) was then cultured
150 in plates of potato dextrose agar (1% Difco PDA; BD company, France) and carboxymethyl
151 cellulose (1% CMC; Sigma Aldrich, USA, with 0.8 % agar; BD company, France) with kanamycin
152 (50 μg/mL; Sigma-Aldrich, USA) and incubated (25 °C) until growth was observed. Colonies
153 exhibiting varied morphologies were purified and transferred onto PDA plates; photographs were
154 taken after incubation (15 days, 30 °C).
155
156 *Molecular identification of the isolated fungi*
157
158 Genomic DNA was extracted from the isolated fungi using the method described by Lodhi
159 *et al.* (1994) with modifications. First, two agar disks (diameter 8 mm) from each fungus were
160 added to a centrifuge tube (2 mL) and were ground with sterile micro-pestles. Extraction buffer

161 (750 µL, sodium EDTA 20 mM, tris-HCl 100 mM, NaCl 1.4 M, CTAB 2 % [w/v], PVP 2% [w/v]
162 and ß-mercaptoethanol 0.2%) was added; the tubes were vortexed and incubated (20 min, 65 °C).
163 For DNA separation, trichloromethane-octanol (750 µL, 24:1) was added to the mixture and
164 centrifuged (25 °C, 14,000 rpm). DNA from the top aqueous phase (600 µL) was precipitated on
165 an addition of 2-propanol (600 µL; Sigma-Aldrich, USA). Then, ethanol (70 %, 500 µL; Sigma-
166 Aldrich, USA) was used to wash the precipitated DNA. Finally, DNA was resuspended in Tris-
167 EDTA buffer (50 µL) with RNAse (1 µL, 10 mg/mL; Thermo Fisher Scientific, USA).

168 To obtain a preliminary identification of the isolates, the nrDNA internal transcribed
169 spacers (ITS) were amplified with primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5
170 (GGAAGTAAAAGTCGTAACAAGG) (White *et al.*1990). Depending on the results from ITS,
171 secondary markers were used to refine the identifications for some isolates, i.e., portions of the
172 translation elongation factor 1-alpha (TEF1; primers CATCGAGAAGTTCGAGAAGG and
173 TACTTGAAGGAACCCTTACC) (Carbone and Kohn 1999) and beta-tubulin (TUB2; primers
174 AACATGCGTGAGATTGTAAGT and TAGTGACCCTTGGCCCAGTTG) (O'Donnell and
175 Cigelnik 1997) genes. Each reaction (total volume 20 µL) consisted of Master Mix (10 µL, 2X;
176 Thermo Fisher Scientific, USA), bovine serum albumin (BSA, 0.5 µL, 20 mg/mL; Sigma Aldrich,
177 USA), dimethyl sulfoxide (DMSO, 1.5 µL; Sigma Aldrich, USA), and primers (0.5 µL each, 10
178 µM) and DNA (2 µL, 50 ng/µL). PCR reactions were implemented in a thermal cycler (9902
179 Veriti, Applied Biosystem, Norwalk, USA), according to conditions described by Schoch *et al.*
180 (2012) for ITS, Carbone and Kohn (1999) for TEF1 and O'Donnell and Cigelnik (1997) for TUB2.
181 Sanger sequencing of PCR products was performed with Psomagen (USA); the raw sequences
182 were edited and assembled in Bioedit v.7.2. Isolate identification was performed by comparing the
183 consensus sequences against the GenBank database using the BLAST search tool. Then, a

184 cladogram was constructed using the ITS sequences. For this, the two closest matches, with type
185 material prioritized, were retrieved/downloaded from the BLAST analysis and aligned using
186 MUSCLE (Edgar 2004). The resulting alignment in Phylip format was submitted to Bayesian
187 Inference analysis with Exabayes (Aberer *et al.* 2014). MCMC was run in parallel and 15 million
188 generations were done with 25% burn-in. All analyses were run in the Kabré supercomputer
189 (CNCA-CONARE, Costa Rica). A consensus tree was visualized and edited with FigTree v.1.4.3
190 (Rambaut 2010). Newly generated sequences were deposited in GenBank under accession
191 numbers ON479855-ON479876 (ITS), ON720280-ON720285 (TEF1), and ON734081–
192 ON734096 (TUB2).
193
194 *Screening of cellulolytic activity*
195
196 The screening of cellulase-producing fungi was undertaken on carboxymethyl
197 cellulose plates (CMC, 1 %; Sigma Aldrich, USA) as the sole carbon source, supplemented with
198 agar (0.8 %, Sigma Aldrich, USA; Johnsen and Krause 2014; Gohel *et al.* 2014). For this purpose,
199 agar disks (diameter ~0.8 mm) of each fungus were placed in the center of CMC plates and
200 incubated (7 days, 30 °C). After incubation, each plate was flooded with Gram's iodine stain (10
201 mL; Sigma-Aldrich, USA; Kasana *et al.* 2008; Gohel *et al.* 2014) and washed with water for 10
202 min. Because Gram's iodine dye is held only by integral cellulose polymers, cellulase activity is
203 revealed by the clear zones appearing as pale halos (Florencio *et al.* 2012). Photographs were taken
204 before and after staining the plates; software ImageJ v.1.52k (Bourne 2010) was used to measure
205 fungal growth (as the diameter of the colony) and the halo diameter for a subsequent calculation

of the enzymatic index (EI), a semiquantitative estimate of enzyme activity according to the following formula (Florencio *et al.* 2012):

$$\mathrm{EI} = \frac{\text{Diameter of hydrolysis zone}}{\text{Diameter of colony}}$$

The experiments were performed in triplicate; *Pleurotus ostreatus* served as a positive control (Garzlllo *et al.* 1994; Valášková and Baldrian 2006).

Results

Material characterization by infrared spectra (ATR-FTIR) and X-ray fluorescence (XRF)

Macroscopically all documents analyzed showed detailed damage as observed in Fig. 1, in which signs of humidity and possible leakage are visible in several areas. Particularly, in the Independence Act surface, orangish spots were present. When observed under UV light, those areas are fluorescent and appear as dark spots in UV reflectance photographs (Supplementary Fig. S1).

The chemical composition of historic documents (both inks and paper) was studied through nondestructive and portable techniques (See Materials and Methods). The composition of each document is shown in Table 1. The composition of the organic substrate was determined by comparing the IR spectra with databases; the elements present in the ink and additives were resolved with X-ray fluorescence. The results showed that the paper in the documents from 1500–1900 was handmade mainly from cotton with watermark presence (excepting the second page from

229 the Cloudy Days Act, which was cellulose-based). In the Political Constitution (1991 replicate),
230 modern paper was used, characterized by shorter fibers and greater lignin content; this indicates
231 that this paper was made from wood cellulose. The results showed that the ink employed in the
232 documents from 1821 was ferrogallic, formed by iron sulfate salts in combination with gallic and
233 tannic acids (Table 1 and Supplementary Fig. S1).
234
235 *Isolation and identification of fungi*
236
237 In total, 22 fungal isolates (Supplementary Fig. S2) were recovered from the Costa Rican
238 historic documents, being the Political Constitution (1991 replicate) that with the most isolates (15
239 in total). The taxonomic identification provided by BLAST and ITS phylogenetic analyses are
240 shown in Table 2 and Fig. 2, respectively. The fungi recovered belong to 14 genera and eight
241 orders. Two taxa were obtained from the Independence Act, two from the Cloudy Days Act, two
242 from the document from 1549, and only one fungal isolate from the oldest document (Guatemalan
243 Series 1539). The phylogenetic placement of the isolates corresponded to eight orders, as shown
244 in the ITS cladogram (Fig. 2). The phylogenetic analysis supports the identifications performed
245 with the BLAST tool, at least at the genus level. TEF1 and TUB2 sequences refined the
246 identification for some of the isolates (Table 2).
247 Most of the fungi found belong in the Ascomycota (86%), followed by Basidiomycota
248 (14%). Among the resulting orders, the majority belong in Hypocreales (23%; *Acremonium,*
249 *Beauveria*, and *Purpureocillium*), Eurotiales (18%; *Aspergillus* and *Penicillium*), and Capnodiales
250 (18%; *Cladosporium*). The Basidiomycota was represented by *Coprinellus, Trametes* and an
251 unidentified species of Psathyrellaceae.

252

253 *Cellulolytic activity*

254

255 The results from cellulolytic activity are shown in Table 3. For the 22 isolates tested, the
256 Enzymatic Index (EI) average was 2.45, with *Cyphellophora* aff. *pluriseptata* CP2-A4P isolated
257 from the Political Constitution being the fungus with the greatest cellulolytic activity (4.0 ± 0.3),
258 followed by *Penicillium steckii* ND1-A1P (3.3 ± 0.3), and *Cladosporium* sp. CP1-A2P (3.3 ± 0.1).
259 In contrast, *Purpureocillium lilacinum* 1539-A1P —from the 1539 Guatemalan Series— was the
260 only isolate without cellulolytic activity. The other fungal isolates showed cellulolytic activity
261 above the levels of the control (*Pleurotus ostreatus*), except for *Trametes* CP1-A3C.

262

263 **Discussion**

264

265 In this work we determined the chemical and microbiological composition of five
266 important historic documents of Costa Rica, including the Independence Act of 1821. Through
267 spectral techniques, we determined that for the documents dated between 1500 and 1900 (i.e., the
268 Cloudy Days Act, the Independence Act of Costa Rica of 1821, and two documents from the
269 Guatemalan Series of 1539 and 1549), the composition of the paper was cotton (i.e., approximately
270 90% cellulose (Felgueiras *et al.* 2021), except the second page of the Cloudy Days Act, which is
271 composed of cellulose acetate. The 1991 replicate of the Political Constitution of 1949 was made
272 of cellulose and lignin; this paper presented the greatest amount of lignin, which indicates a modern
273 paper made from wood (hereafter referred to as wood cellulose-based paper).

274 Despite that modern paper is relatively stable, deterioration is common with increased
275 levels of humidity and acidification produced by oxidation and the presence of microorganisms
276 (Proniewicz *et al.* 2001). Although the 1991 replicate of the Political Constitution from 1949 is
277 the most recent, 15 fungal isolates were obtained, whereas from all the oldest documents (made
278 mainly of cotton), seven in total were obtained. These data suggest that wood-cellulose-based
279 paper possesses characteristics that are more suitable for fungal colonization than cotton-based
280 documents. These observations make sense if we consider the differences between the chemical
281 composition of cotton and paper obtained from other plant fibers such as wood. Cotton contains
282 approximately 90% cellulose (hence it is sometimes referred to as highly pure cellulose), whereas
283 other natural fibers, such as wood, contain 40–55% cellulose combined with other constituents
284 such as lignin and hemicelluloses (Felgueiras *et al.* 2021). Cellulose is considered a two-phase
285 material, having both crystalline and amorphous phases (Ling *et al.* 2019). Cotton cellulose fibers
286 are reported to have a greater degree of polymerization and crystallinity, which generates stronger
287 fibers and greater resistance to hydrolysis and biodegradation. (Itävaara *et al.* 1999). Therefore,
288 these two characteristics (higher cellulose content and higher crystallinity) make cotton a substrate
289 that is less prone to microbial colonization than a material such as wood-based paper, which is the
290 case of the paper of the 1991 replicate of the Political Constitution of 1949. Enzymatically, the
291 reduced ability for microbial colonization of cotton-based papers is related to the more limited
292 access of the cellulase enzyme complex to the substrate due to the orderly and compact architecture
293 of the crystalline cellulose present in cotton (Arantes and Saddler 2010).

294 In Fig. 1C, the orangish spots over the Independence Act surface indicate oxidation from
295 cellulose and iron, probably caused by both abiotic and biotic factors (Choi 2007). Those areas are
296 fluorescent under UV light and appear as dark spots in UV reflectance photographs

297 (Supplementary Fig. S1). Although excessive humidity can itself trigger oxidation, contributing to
298 the damage of important documents, the inks used can also be affected by this abiotic factor,
299 producing the migration of metal ions of common ink components such as iron and copper,
300 compromising the preservation of historic and cultural heritage due to weakened paper (Henniges
301 *et al.* 2006). Our work determined that the ink employed in the documents from 1821 was
302 ferrogallic, formed by iron sulfate salts in combination with gallic and tannic acids. The last was
303 confirmed from the XRF and multispectral photography (see Supplementary Fig. S1). Ink of this
304 kind is visible under infrared and, in darkness, under UV light (Havermans *et al.* 2003). The
305 implementation of optical spectroscopy techniques, such as those used in this work, have been
306 shown to help identify the early stages of document damage by microorganisms such as fungi,
307 relating changes in the spectral composition to the active presence of fungi (Povolotckaia *et al.*
308 2019).

309 In the historic documents, we obtained a total of 22 fungi belonging to 14 genera, of which
310 five (35%) were previously identified in paper-based historic documents (Bensch *et al.* 2018;
311 Pinheiro *et al.* 2019; Romero *et al.* 2021; Trovão and Portugal 2021). The presence of two
312 Chaetothyriales members (isolates 1549-4A1C and CP2-A4P) is interesting because they are
313 inhabitants of environments with limited resources, such as rocks, insects, and ant nests; some
314 species in the order can even become pathogenic for humans, especially in tropical regions (Attili-
315 Angelis *et al.* 2014; Ahmed *et al.* 2021). *Cyphellophora,* also Chaetothyriales, is closely related to
316 *Phialophora* (Feng *et al.* 2014), which includes species that grow in extremely acidic conditions
317 and have been reported to produce ß-mannanase and ß -glucanase enzymes (Zhao *et al.* 2010; Zhao
318 *et al.* 2012). Our isolate CP2-A4P had the greatest enzymatic index value (4.0 ± 0.3), for which
319 further analysis involving enzyme identification could yield intriguing results. Isolate *Penicillium*

320 *steckii* ND1-A1P originated from a cotton-based substrate and presented an enzymatic index of
321 3.3 ± 0.3. This corresponds to what is commonly observed because *Aspergillus* and *Penicillium*
322 species are registered continuously in paper biodeterioration and are known to break the hydrogen
323 bonds, which translates into a weakening of the documents, regardless of their composition
324 (Povolotckaia *et al.* 2019). In total, three isolates corresponding to *Penicillium* were obtained from
325 the Cloudy Days Act, the Political Constitution, and the 1549 Guatemalan Series. We registered
326 only a single isolate corresponding to *Aspergillus hiratsukae* (AI3-A1P), which was recovered
327 from the Independence Act of 1821. This was unexpected because *Aspergillus* spp. are frequent
328 biodeteriogens in cultural heritage objects and spaces in which historic documents are preserved,
329 such as the National Archive of Cuba, in which *Aspergillus*, *Cladosporium,* and *Penicillium* were
330 the most frequent airborne genera reported (Borrego and Perdomo 2016). In this work,
331 *Cladosporium* isolates were present in the Cloudy Days Act (isolate ND2-A1P), the Independence
332 Act (isolate AI1-A1C), and the Political Constitution (isolates CP1-A1P and CP1-A2P),
333 corresponding to cellulose acetate, cotton, and wood cellulose substrates. *Cladosporium* spp. are
334 reported as colonizers in agricultural waste (Herculano *et al.* 2011), artworks (Coronado-Ruiz *et*
335 *al.* 2018), repositories of historic documents (Borrego and Perdomo 2016), and as extremophiles
336 in high-altitude tropical glaciers (Calvillo-Medina *et al.* 2020).

337 *Neopestalotiopsis clavispora* (CP1-A2C) and *Pestalotiopsis kenyana* (CP2-A2C)
338 (Xylariales), both obtained from the Political Constitution, belong to a genus known as a common
339 endophyte, saprotroph, and phytopathogen in diverse plants and climates (Reddy *et al.* 2016).
340 Because of the various ecological relationships this genus has with plants, it has attracted attention
341 for its cellulolytic abilities, with over 400 possible enzymes found through the study of a
342 *Pestalotiopsis* isolated from a mangrove (Arfi *et al.* 2013). So far, there are some cellulolytic

343 enzymes known from this genus that have been studied, such as the xylanase (Koh *et al.* 2021) or
344 the cellulases (Goukanapalle *et al.* 2020). Another single isolate obtained from the Political
345 Constitution was *Periconia* CP1-A1C, which belongs to the dark septate fungi Pleosporales, with
346 some isolates reported with thermostable β-Glucosidases suitable for biotechnological processes
347 (Harnpicharnchai *et al.* 2009). *Periconia* species have been encountered in extreme environments,
348 such as deserts, seas, tropical glaciers (Calvillo-Medina *et al.* 2020), and even a new species
349 growing over lithographs from the 19th century (Coronado-Ruiz *et al.* 2018), among others.

350 Hypocreales isolates were only found in the Political Constitution. Intriguing were two
351 isolates known as insect pathogens, i.e., *Beauveria* (CP2-A3P) and *Purpureocillium* (1539-A1P
352 and CP2-A1P; Shrestha *et al.* 2019). The cellulolytic activity recorded for our *Beauveria* isolate
353 was 3.1 ± 0.3, which is consistent with reports of cellulolytic activity driven by a thermally stable
354 β-Glucosidase in *Beauveria bassiana* (Borgi and Gargouri 2016). Screening for cellulolytic
355 activity in this entomopathogenic species is not commonly done because it is mainly studied for
356 its biological control abilities in multiple agricultural crops (Posada and Vega 2006; Sanjuan *et al.*
357 2014; Mwamburi 2021). *Beauveria* has also been found as an endophyte, along with
358 *Purpureocillium* (Kepler *et al.* 2013). An isolate of *Purpureocillium lilacinum* has been reported
359 as a biodeteriogen of indoor materials able to grow in alkaline materials, producing damage in
360 limestones and plasters of cultural heritage in Russia (Ponizovskaya *et al.* 2019). The tolerance to
361 extreme conditions by some *Purpureocillium* spp. results in the ability to become pathogenic to
362 humans and resistant to fungicides (Calvillo-Medina *et al.* 2020). Although one isolate recovered
363 from the 1539 Guatemalan Series (cotton-based) was unable to grow in the carboxymethyl
364 cellulose media, isolate CP2-A1P from the Political Constitution (wood cellulose-based) attained
365 EI of 3.2. *Acremonium* isolates CP2-A3C and ND2-A1P registered enzymatic indexes < 3.

366 Previous researchers reported a xylanase produced by *Acremonium cellulolyticus* (Watanabe *et al.*
367 2014); another group subsequently revised the taxonomy and concluded a misidentification of an
368 *Acremonium* species, reidentifying isolate Y-94 from Japan as *Talaromyces* (Eurotiales) (Fujii *et*
369 *al.* 2014). Apart from the latter, only a xylanase has been reported for *A. alcalophilum* (Šuchová
370 *et al.* 2020).

371 Small enzymatic indexes also occurred for three basidiomycetes recovered from the
372 Political Constitution (CP1-A3C, CP1-A3P, and CP2-A2P). These were identified as belonging to
373 Psathyrellaceae, including *Coprinellus* (CP2-A2P). From these, only one report exists of a
374 xylanase produced by *Coprinellus disseminatus,* which was tolerant to varied pH and temperatures
375 (Agnihotri *et al.* 2010). Isolate *Trametes* CP1-A3C registered an EI less than the control (*Pleurotus*
376 *ostreatus*). Species with tough fruiting bodies, such as *Trametes maxima,* are reported to possess
377 laccase activity, even when exposed to herbicides (Cupul *et al.* 2014); an isolate of *T. versicolor*
378 is reported to cause the effective degradation of fungicides (Rodríguez-Rodríguez *et al.* 2012).
379

380 **Conclusions**

381

382 Regardless of the material of a document of origin —cotton or wood cellulose— most
383 recovered fungal isolates presented cellulolytic activity. From the historic documents sampled, the
384 Political Constitution had the greatest number of isolates, which suggests that wood cellulose-
385 based paper possesses characteristics more suitable for fungal colonization than the oldest cotton-
386 based documents (i.e., documents from 1500–1900). Cotton contains 90% cellulose and has great
387 crystallinity, which makes it more difficult for the enzymatic machinery of microorganisms to
388 degrade these polymers and use them for their nutritional requirements. Even though the oldest

389 documents (e.g., Independence Act and the Cloudy Days Act) yielded few isolates, restoration and
390 improvement of the conditions they are stored in should be implemented to avoid oxidation and
391 weakening of their fibers which could then increase microbiological contamination. The results of
392 our work provide valuable information for establishing the appropriate protocols to undertake that
393 restoration and conservation. Determining the chemical composition of the paper and the
394 composition of the inks, as well as the microbiological load, allows for identification of the most
395 appropriate strategies and treatments to restore documents as important to Costa Rica as the Act
396 of Independence itself. Because historic documents can be considered microhabitats with limited
397 resources, the screening of species with novel biotechnological applications in such environments
398 is a promising and fascinating field. The study of how best to conserve historic documents is vital
399 to preserve, in a satisfactory condition, important sources and records of human history.
400 Multidisciplinary approaches such as the present work can help curators make the best choice of
401 restoration techniques and eventually fulfill the Korean phrase: "Silk can stand five hundred years,
402 but paper can stand one thousand" (Jeong et al., 2014).
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683 **Tables**

684

685 **Table 1. Chemical, substrate, and ink characterization of the historic documents from Costa**

686 **Rica.**

Document	**Sections analyzed for chemical characterization**	**Sections analyzed for fungal isolation**	**Paper composition**	**Ink chemical elements detected**
Independence Act, 1821	Pages 1-3	Pages 1-3	Cotton	Fe, Ca, Zn, K
Cloudy Days Act, 1821	Page 1 Page 2	Full document	Cotton Cellulose acetate	Fe, Ca, Zn, K, S, Cl, Pb Fe, Ca, Zn
Political Constitution, 1949 (1991 reproduction)	Full document	Cover and Slavery Abolition page	Wood cellulose	Not determined
Guatemalan Series 1539	Full document	Pages 1,8,3,17	Cotton	Not determined
Guatemalan Series 1549	Full document	Pages 2,5,9,10	Cotton	Not determined

687

688

689 **Table 2.** Identification (ID); BLAST results using ITS, TEF1 and TUB2; and origin of documents. Names in bold indicate the
690 assigned classification.
691

Isolate number	**Origin**	**Closest match and accession number / % similarity ***		
		ID with ITS	**ID with TEF1**	**ID with TUB2**
1539-A1P	Guatemalan series, 1539	***Purpureocillium lilacinum*** MZ359582 / 99.3	***Purpureocillium lilacinum*** MH613753 / 100	***Purpureocillium lilacinum*** JQ965112 / 99.8
1549-1A1P	Guatemalan series, 1549	***Penicillium* sp**. FJ752622 / 99.81	**-**	***Penicillium compactum*** KM973202 / 98.6
1549-4A1C		**Unidentified Herpotrichiellaceae** KJ612089 / 90.48	no match	no match
AI1-A1C	Independence Act, 1821	***Cladosporium* sp**. OK274323 / 100	-	***Cladosporium oxysporum*** EF101455 / 95.5
AI3-A1P		***Aspergillus hiratsukae*** MN347034 / 100	-	***Aspergillus hiratsukae*** MH644026 / 100
CP1-A1C	Political Constitution, 1949 (1991 reproduction)	***Periconia* sp**. KP128003 / 99.80	no match	no match
CP1-A1P		***Cladosporium* sp.** OK274323 / 92.48	-	***Cladosporium* sp.** JQ217373 / 97
CP1-A2C		***Pestalotiopsis microspora*** OK254042 / 100	-	***Neopestalotiopsis clavispora*** OM328818 / 98.2
CP1-A2P		***Cladosporium* sp.** EF504401 / 100	-	-
CP1-A3C		***Trametes hirsuta*** GQ280373 / 100	-	-
CP1-A3P		**Unidentified Psathyrellaceae** JQ922137 / 100	-	no match
CP2-A1C		**Unidentified Pleosporales** KP263091 / 92.42	-	***Biatriospora* sp**. MF588919 / 86

CP2-A1P		***Purpureocillium lilacinum*** MZ359582 / 99.88	-	***Purpureocillium lilacinum*** GU968702 / 100%
CP2-A2C		***Pestalotiopsis trachycarpicola*** MZ453106 / 99.82	***Neopestalotiopsis* sp.** KR493607 / 100	***Pestalotiopsis kenyana*** KX895360 / 100
CP2-A2P		***Coprinellus* sp.** MK307658 / 99.84	-	-
CP2-A3C		***Acremonium persicinum*** JQ599382 / 99.42	-	-
CP2-A3P		***Beauveria* aff. *bassiana*** MZ618707 / 100	-	***Xenoacremonium recifei*** KM232105 / 89
CP2-A4C		***Acremonium persicinum*** JQ599382 / 100	-	-
CP2-A4P		***Cyphellophora* aff. *pluriseptata*** MH063042.1 / 91.7	-	***Cyphellophora* sp.** LR814116 / 80%
CP2-A5C		***Penicillium* aff. *sumatraense*** MH864547 / 100	-	-
ND1-A1P	Cloudy Days Act, 1821	***Penicillium steckii*** MZ568311 / 100	-	***Penicillium steckii*** MW196656 / 100
ND2-A1P		***Cladosporium* sp.** OK242741 / 100	-	***Cladosporium* aff. *oxysporum*** KU216745 / 99.7

692 *If the percent identity is less than 80% and query coverage less than 50%, then the result is indicated as “no match.”
693

694 **Table 3.** Enzymatic index (EI) registered by fungal isolates (± indicates standard deviation based

695 on three replicates).

Isolate code	Taxonomy	Enzymatic Index (EI)
CP2-A4P	*Cyphellophora* aff. *pluriseptata*	4.0 ± 0.3
ND1-A1P	*Penicillium steckii*	3.3 ± 0.3
CP1-A2P	*Cladosporium* sp.	3.3 ± 0.1
CP2-A1P	*Purpureocillium lilacinum*	3.2 ± 0.0
1549-4A1C	Herpotrichiellaceae	3.1 ± 0.4
CP2-A2C	*Pestalotiopsis kenyana*	3.1 ± 0.5
CP2-A3P	*Beauveria* aff. *bassiana*	3.1 ± 0.3
CP1-A1P	*Cladosporium* sp.	3.0 ± 0.1
CP2-A5C	*Penicillium* aff. *sumatraense*	2.9 ± 0.7
CP2-A1C	Pleosporales	2.8 ± 0.2
AI3-A1P	*Aspergillus hiratsukae*	2.8 ± 0.0
1549-1A1P	*Penicillium compactum*	2.6 ± 0.9
AI1-A1C	*Cladosporium* sp.	2.6 ± 0.1
CP1-A1C	*Periconia* sp.	2.5 ± 0.3
ND2-A1P	*Cladosporium* aff. *oxysporum*	2.5 ± 0.2
CP1-A3P	Psathyrellaceae	2.2 ± 0.2
CP2-A2P	*Coprinellus* sp.	2.0 ± 0.3
CP2-A4C	*Acremonium persicinum*	1.8 ± 0.1
CP1-A2C	*Neopestalotiopsis clavispora*	1.7 ± 0.1
CP2-A3C	*Acremonium persicinum*	1.6 ± 0.2
Control	*Pleurotus ostreatus*	1.3 ± 0.1
CP1-A3C	*Trametes hirsuta*	1.1 ± 0.0
1539-A1P	*Purpureocillium lilacinum*	0

Figure captions

Figure 1. Historic documents from Costa Rica analyzed for chemical and substrate characterization. **A,B**. Independence Act. **C**. Signs of deterioration on the Independence Act, including yellow spots around the letters. **D**. Cloudy Days Act. **E**. Humidity mark on Cloudy Days Act. **F**. Oxidation signs around the ink from Cloudy Days Act. **G**. 1949 Political Constitution

(1991 reproduction). **H**. Signs of microbiological contamination on 1949 Political Constitution (1991 reproduction). **I**. Yellow spots on 1949 Political Constitution (1991 reproduction). **J**. Fragment of the 1539 Guatemalan Series. **K**. Fragment of the 1549 Guatemalan Series. **L,M**. Signs of leakage and humidity on 1539-1549 Guatemalan Series.

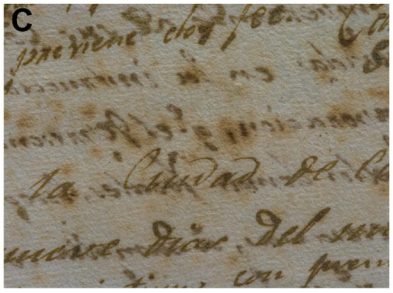
Figure 2. Bayesian Inference consensus cladogram based on nrDNA ITS sequences. Posterior probabilities are indicated at branches. LnL = -13564.21

Independence Letter 1821

A

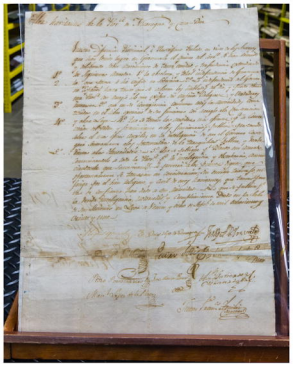
B

C

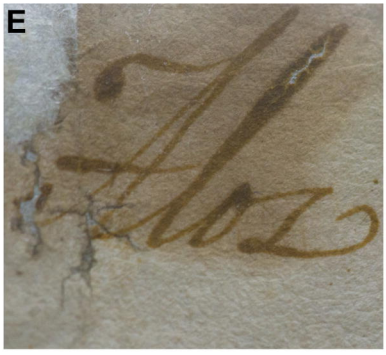


Cloudy Days Letter 1821

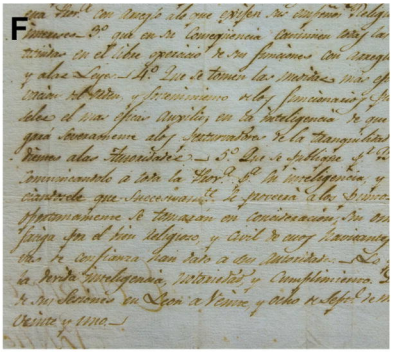
D



E

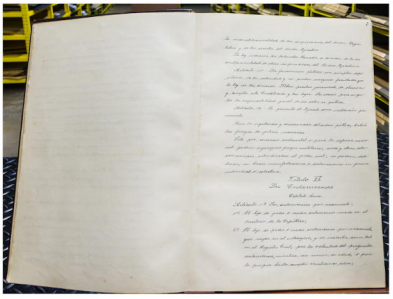


F



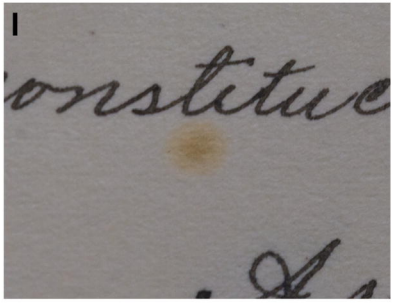
Political Constitution 1991 reproduction

G



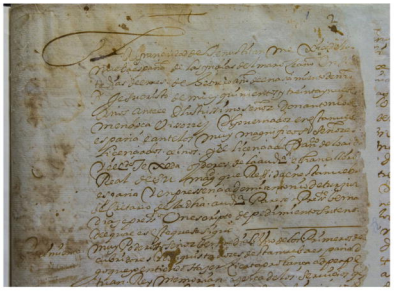
H

I

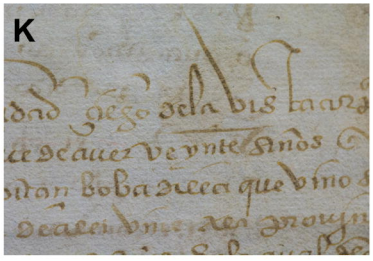


Guatemalan Series 1539 and 1549

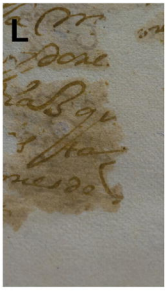
J



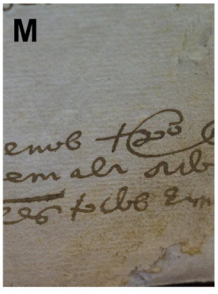
K

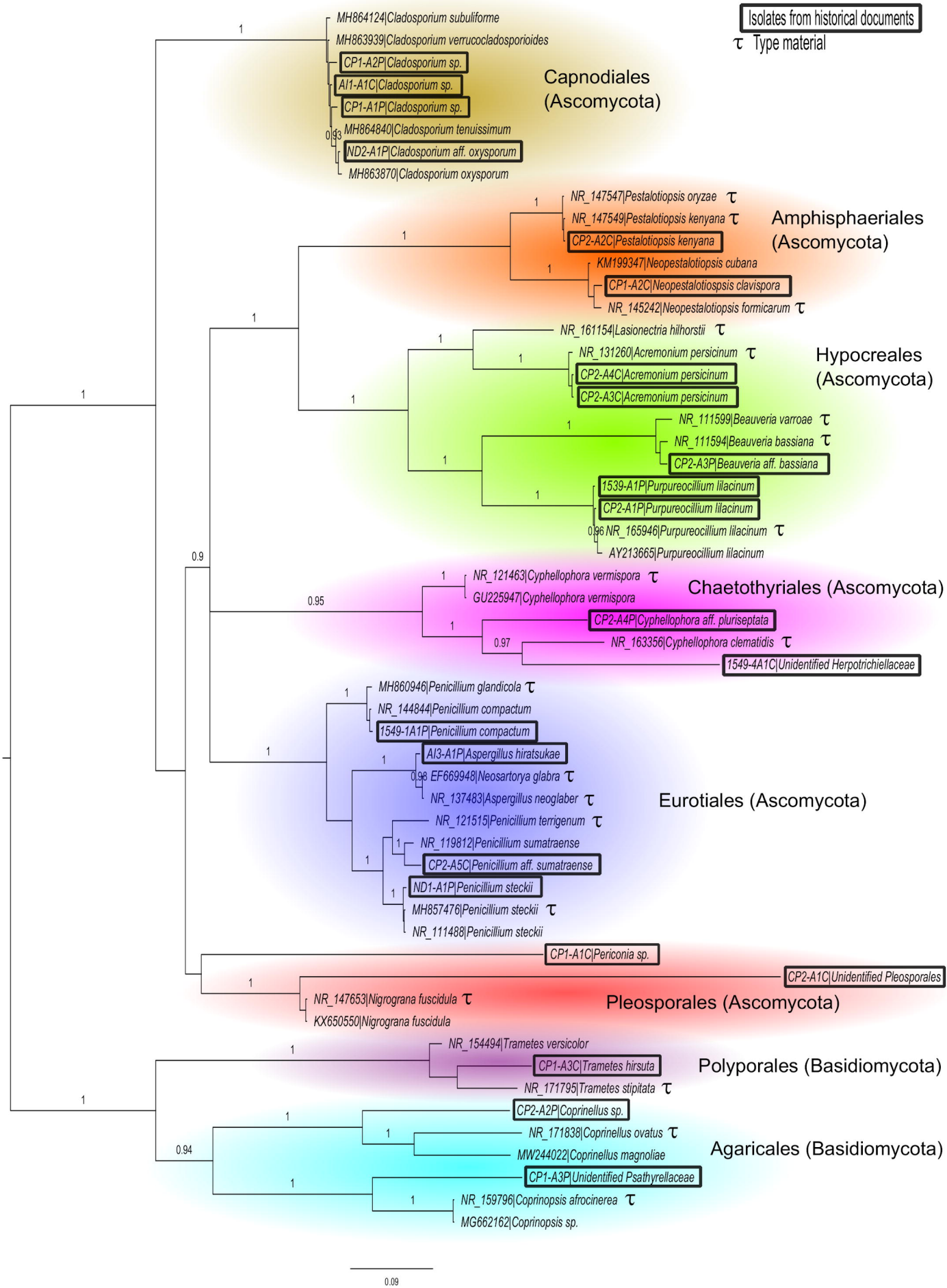


L



M




Isolates from historical documents
τ Type material
MH864124|Cladosporium subuliforme
MH863939|Cladosporium verrucocladosporioides
CP1-A2P|Cladosporium sp.
AI1-A1C|Cladosporium sp.
CP1-A1P|Cladosporium sp.
MH864840|Cladosporium tenuissimum
ND2-A1P|Cladosporium aff. oxysporum
MH863870|Cladosporium oxysporum
Capnodiales (Ascomycota)
NR_147547|Pestalotiopsis oryzae τ
NR_147549|Pestalotiopsis kenyana τ
CP2-A2C|Pestalotiopsis kenyana
KM199347|Neopestalotiopsis cubana
CP1-A2C|Neopestalotiospsis clavispora
NR_145242|Neopestalotiopsis formicarum τ
Amphisphaeriales (Ascomycota)
NR_161154|Lasionectria hilhorstii τ
NR_131260|Acremonium persicinum τ
CP2-A4C|Acremonium persicinum
CP2-A3C|Acremonium persicinum
NR_111599|Beauveria varroae τ
NR_111594|Beauveria bassiana τ
CP2-A3P|Beauveria aff. bassiana
1539-A1P|Purpureocillium lilacinum
CP2-A1P|Purpureocillium lilacinum
NR_165946|Purpureocillium lilacinum τ
AY213665|Purpureocillium lilacinum
Hypocreales (Ascomycota)
NR_121463|Cyphellophora vermispora τ
GU225947|Cyphellophora vermispora
CP2-A4P|Cyphellophora aff. pluriseptata
NR_163356|Cyphellophora clematidis τ
1549-4A1C|Unidentified Herpotrichiellaceae
Chaetothyriales (Ascomycota)
MH860946|Penicillium glandicola τ
NR_144844|Penicillium compactum
1549-1A1P|Penicillium compactum
AI3-A1P|Aspergillus hiratsukae
EF669948|Neosartorya glabra τ
NR_137483|Aspergillus neoglaber τ
NR_121515|Penicillium terrigenum τ
NR_119812|Penicillium sumatraense
CP2-A5C|Penicillium aff. sumatraense
ND1-A1P|Penicillium steckii
MH857476|Penicillium steckii τ
NR_111488|Penicillium steckii
Eurotiales (Ascomycota)
CP1-A1C|Periconia sp.
CP2-A1C|Unidentified Pleosporales
NR_147653|Nigrograna fuscidula τ
KX650550|Nigrograna fuscidula
Pleosporales (Ascomycota)
NR_154494|Trametes versicolor
CP1-A3C|Trametes hirsuta
NR_171795|Trametes stipitata τ
Polyporales (Basidiomycota)
CP2-A2P|Coprinellus sp.
NR_171838|Coprinellus ovatus τ
MW244022|Coprinellus magnoliae
CP1-A3P|Unidentified Psathyrellaceae
NR_159796|Coprinopsis afrocinerea τ
MG662162|Coprinopsis sp.
Agaricales (Basidiomycota)
0.09