



HAL
open science

Leptobacillium cavernicola, a newly discovered fungal species isolated from several Paleolithic-decorated caves in France

Johann Leplat, Alexandre Francois, Faisl Bousta

► **To cite this version:**

Johann Leplat, Alexandre Francois, Faisl Bousta. Leptobacillium cavernicola, a newly discovered fungal species isolated from several Paleolithic-decorated caves in France. *Phytotaxa*, 2022, 571 (2), pp.186-196. 10.11646/phytotaxa.571.2.5 . hal-04615611

HAL Id: hal-04615611

<https://hal.science/hal-04615611>

Submitted on 18 Jun 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 ***Leptobacillium cavernicola*, a newly discovered fungal species isolated from**
2 **several Paleolithic-decorated caves in France**

3

4 JOHANN LEPLAT^{1,2,3*}, ALEXANDRE FRANCOIS^{1,2,4}, FAISL BOUSTA^{1,2,5}

5 ¹ *Laboratoire de Recherche des Monuments Historiques (LRMH), Ministère de la Culture, 29*
6 *rue de Paris, 77420 Champs-sur-Marne, France*

7 ² *Sorbonne Universités, Centre de Recherche sur la Conservation (CRC, USR 3224), Museum*
8 *national d'Histoire naturelle, Ministère de la Culture, CNRS; CP21, 36 rue Geoffroy-Saint-*
9 *Hilaire, 75005 Paris, France*

10 ³ johann.leplat@culture.gouv.fr; <https://orcid.org/0000-0002-1288-0397>

11 ⁴ alexandre.francois@culture.gouv.fr; <https://orcid.org/0000-0002-1706-5084>

12 ⁵ faisl.bousta@culture.gouv.fr; <https://orcid.org/0000-0002-1056-8463>

13 *Corresponding author: johann.leplat@culture.gouv.fr

14 **Abstract**

15

16 Several interesting fungal strains were recovered from air and surface samplings in a number of
17 Paleolithic-decorated caves in France during multiple surveys. Multi-locus (ITS, LSU, SSU,
18 RPB1, RPB2 and TEF-1 α) phylogenetic analysis of these strains through maximum likelihood
19 and Bayesian inference coupled with a morphological characterization revealed a new fungal
20 taxa, *Leptobacillium cavernicola* sp. nov. This new species morphologically and
21 phylogenetically close to *Leptobacillium muralicoa*, which was also first isolated from a cultural
22 heritage context. The study of this new species led us to reconsider the taxonomic position of
23 strains that have hitherto been identified as *Leptobacillium* spp., suggesting that several new
24 species could be added to the genus.

25

26 **Keywords**

27

28 Cordycipitaceae, cultural heritage, *Leptobacillium* genus, new fungal taxa

29

30 **Introduction**

31

32 The genus *Leptobacillium* was introduced by (Zare & Gams 2016) during the revision of the
33 former *Verticillium* Nees section *Albo-erecta*, which was clearly polyphyletic. The section
34 contained white or whitish species with verticillium-like, erect conidiophores. The new genus
35 *Leptobacillium* accommodated the strains identified until then as *Verticillium leptobactrum* W.
36 Gams, which phylogenetically belong to the Cordycipitaceae family. Zare & Gams (2016)
37 initially described the single species *L. leptobactrum*, composed of the two varieties *L.*
38 *leptobactrum* var. *leptobactrum* and *L. leptobactrum* var. *calidius*, which are distinguished by

39 their optimal growth temperature. The name of the genus refers to its characteristically narrow
40 microconidia. The genus currently contains six species. *Simplicillium* Zare & W. Gams is
41 phylogenetically the closest genus to *Leptobacillium* in the Cordycipitaceae family (Zare &
42 Gams 2016). Thus, several *Leptobacillium* species were first described as *Simplicillium* species
43 before being moved to the *Leptobacillium* genus, i.e. *L. chinense* (F. Liu & L. Cai) Okane,
44 Nonaka, Kurihara, J.P. Abe & Yamaoka, *L. coffeaneum* (A.A.M. Gomes & O.L. Pereira) Okane,
45 Nonaka, Kurihara, J.P. Abe & Yamaoka and *L. filiforme* (R.M.F. Silva, R.J.V. Oliveira, C.M.
46 Souza-Motta; J.L. Bezerra & G.A. Silva) W.H. Chen, Y.F. Han, J.D. Liang, Z.Q. Liang.
47 Many species in the family Cordycipitaceae are known to be entomogenous (Mongkolsamrit *et*
48 *al.* 2018; Wang *et al.* 2020). *Simplicillium*, the sister genus of *Leptobacillium*, is thus known to
49 include not only several entomogenous species, but also fungicolous species (Zare & Gams 2001;
50 Chen *et al.* 2019). Similarly, several reference species in the genus *Leptobacillium* were isolated
51 from insects and fungi, but also from plants and freshwater, or from mural paintings (Okane *et*
52 *al.* 2020; Zare & Gams 2016; Crous *et al.* 2018; Liu & Cai 2012; Gomes *et al.* 2018; Sun *et al.*
53 2019). This last isolation source is interesting because *V. leptobactrum*, the basionym of *L.*
54 *leptobactrum*, was found to be particularly abundant on rock substrates (Daghino *et al.* 2009).
55 This species has also been abundantly studied for its nematophagous properties (Gams 1988;
56 Regaieg *et al.* 2011).
57 The phylogenetic study of reference strains belonging to the genus *Leptobacillium* was based
58 solely on the nuclear ribosomal internal transcribed spacer (ITS) region and the large subunit of
59 nuclear encoded ribosomal DNA (LSU). Yet, several other DNA loci are currently used to study
60 the species in the Cordycipitaceae family (Kepler *et al.* 2017). Consequently, there are very few
61 *Leptobacillium* sequences for the small subunit of nuclear encoded ribosomal DNA (SSU) and
62 the translation elongation factor 1-alpha gene (TEF1- α) in the NCBI GenBank database (Clark
63 *et al.* 2016), while no sequences are available for the largest subunit of RNA polymerase II

64 (RPB1) and the second largest subunit of RNA polymerase II (RPB2). In fact, only the ITS
65 sequence is available for the most of the strains available in the database. In this study, we
66 described a new species in the genus *Leptobacillium* on the basis of morphological and
67 phylogenetic features. All the specimens of this new species were isolated from Paleolithic
68 decorated caves. The phylogenetic study of this new species was performed on the complete set
69 of DNA loci currently used in the Cordycipitaceae family to provide new data about
70 *Leptobacillium* genus. This study led to a general discussion about *Leptobacillium* genus and the
71 strains identified as part of this genus.

72

73 **Materials & Methods**

74

75 *Collection and isolation*

76 The eleven studied strains were isolated from four Paleolithic-decorated caves in October 2016
77 or October 2018 (Table 1). Ten of these eleven strains were isolated through air samplings, while
78 the last strain was isolated through swab sampling on the cave wall. The airborne strains were
79 collected with a Duo SAS Super 360 air sampler (VWR-pbi, Milan, Italy). Fifty liters of air were
80 collected at each sampling point using a 219-hole impactor containing malt extract agar (MEA;
81 Merck KGaA, Darmstadt, Germany) culture media in 55 mm Petri dishes. The Petri dishes
82 containing impacted media were then taken to the laboratory for analysis. The plates were
83 incubated in an IPP55 incubator (Mettler GmbH + Co. KG, Büchenbach, Germany) for seven
84 days at 24 °C. Fungi were then isolated from each other using the same culture conditions as
85 those described before. The strain isolated through swabbing followed the same laboratory
86 process. The strains were preserved on MEA under paraffin oil at 4 °C prior to use. Two of the
87 eleven strains preserved in the collection did not grow back from the collection, but their DNA

88 had already been extracted. The phenotypic characterization was, therefore only studied on nine
89 strains, while the phylogenetic study was performed on all eighteen strains.

90

91 *Reference strains*

92 In the phylogenetic study aiming to identify the *Leptobacillium* strains isolated in this study, the
93 reference strains were *Leptobacillium* spp. strains for which sequences from at least two different
94 DNA loci were available in the NCBI GenBank database (Table 2). Numerous strains for which
95 available data concerned only one DNA loci were then added in a second phylogenetic study
96 seeking to study the diversity of available sequences. *Gamszarea wallacei* (H.C. Evans) Z.F.
97 Zhang & L. Cai (CBS 101237) is often used when studying *Leptobacillium* genus, and was thus
98 chosen as an outgroup (Okane *et al.* 2020; Chen *et al.* 2021). The names of the fungal species
99 were updated with the current names of the species, as defined in the MycoBank (Crous *et al.*
100 2004).

101

102 *Morphological study*

103 The macroscopic features were assessed on MEA and on Potato Dextrose Agar (PDA, VWR
104 International, Radnor, USA) after a 14-day incubation at 25 °C. The microscopic features were
105 assessed on MEA after a 14-day incubation at 25 °C. Observations were performed with a
106 Jenavert optical microscope (Zeiss, Oberkochen, Germany) after treatment with lactophenol
107 cotton blue solution (Pro-Lab Diagnostics, Richmond Hill, Canada). Microscopic features were
108 captured with an Lt365R digital camera (Teledyne Lumenera, Ottawa, Canada) using the
109 Archimed program (Microvision Instruments, Evry, France).

110

111

112 *DNA extraction, PCR amplification and sequencing*

113 Fungal DNA was extracted as described by Edel *et al.* (2001). Appropriate primers (Table 3)
114 were used to amplify ITS, LSU, SSU, TEF1- α , RPB1 and RPB2 regions. PCR was performed in
115 25 μ l reactions, with 1 μ l of template DNA, 1 U of Taq DNA polymerase (Invitrogen, Carlsbad,
116 USA), 2.5 μ l of 10X Taq DNA polymerase buffer, 1 μ l of 2 mmol l⁻¹ dNTPs (Thermo Fisher
117 Scientific, Waltham, USA) and 1.5 μ l of each 10 μ mol l⁻¹ primer (Eurogentec, Seraing, Belgium).
118 Amplifications were performed on a PrimeG thermocycler (Bibby Scientific, Stone, UK) using
119 the following parameters: a 5-minute step at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1
120 min at the appropriate annealing temperature for each primer pair (Table 3), 1 min at 72 °C, and
121 a final 10-minute extension step at 72 °C. PCR products were sequenced by Genoscreen (Lille,
122 France) using the same primer set. The generated sequences were submitted to the NCBI
123 GenBank database.

124
125 *Sequence alignment and phylogenetic analyses*

126 The sequences generated in this study were verified with BioEdit v. 7.2.5 (Hall 1999). The
127 sequences related to reference strains were downloaded from the NCBI GenBank database.
128 Multiple sequence alignments for each studied DNA region were performed using the FFT-NS-
129 i alignment strategy from the MAFFT v. 7 web server (Katoh *et al.* 2019). The six previously
130 described DNA loci were used in the study that used reference strains for which at least two
131 different DNA loci were available in the NCBI GenBank database, while only ITS and LSU loci
132 were used in the study that included all available strains. The uninformative gaps and ambiguous
133 regions were removed using the Gblocks program v. 0.91.1 implemented in the NGPhylogeny.fr
134 service, with the program set for a lightly stringent selection (Minimum number of sequences for
135 a conserved position: 50 % of the sequences + 1; Minimum length of a block: 5; Allowed gap
136 position: “with half”;(Talavera & Castresana 2007; Lemoine *et al.* 2019)). The maximum

137 likelihood (ML) analyses of concatenated regions were performed using the graphical interface
138 of RAxML v. 8 set to 1000 bootstrap iterations and the GTRGAMMA substitution model
139 (Stamatakis 2014; Edler *et al.* 2019). Bayesian inference (BI) analyses of concatenated regions
140 were performed using MrBayes v. 3.2.6 implemented in PhyloSuite v. 1.2.1, based on a Markov
141 Monte Carlo Chain (MCMC) set to two simultaneously executed runs for 10,000,000 generations
142 with the GTRGAMMA substitution model (Ronquist *et al.* 2012; Zhang *et al.* 2020a). Trees were
143 sampled every 1000 generations, with the burning fraction set to 25 %. Generated trees were
144 edited using MEGA X (Kumar *et al.* 2018).

145

146 **Results**

147

148 In the phylogenetic study of *Leptobacillum cavernicola* among *Leptobacillum* genus according
149 to reference strains for which at least two different DNA loci were available in the NCBI
150 Genbank database, the same results were obtained for the maximum likelihood and the Bayesian
151 inference strategies (Fig. 1). *Leptobacillum cavernicola* strains constituted a distinctive clade in
152 the genus, *L. muralicola* being the closest species. This result was well supported by bootstrap
153 values and posterior probabilities. The differences observed between *L. cavernicola* strains were
154 scarce for the six DNA loci considered. The studies also highlighted the phylogenetic proximity
155 between *L. filiforme* and *L. symbioticum* species. The phylogenetic studies placed two strains in
156 unexpected clades, namely *L. leptobactrum* var. *calidius* CBS 703.86 and *L. leptobactrum* var.
157 *leptobactrum* CBS 771.69: the former was placed in the *L. chinense* clade and the latter was
158 placed in the newly defined *L. cavernicola* clade.

159 The phylogenetic study of *Leptobacillum cavernicola* in comparison to all the other
160 *Leptobacillum* strains available in the NCBI GenBank database also showed that *L. cavernicola*
161 had probably already been isolated in the past, but had been sorted as *L. leptobactrum* (Fig. 2).

162 Interestingly, several of these potential strains of *L. cavernicola* were also recovered from a cave
163 context. This study also highlighted that numerous strains that were initially identified as *L.*
164 *leptobactrum* should be re-examined in the light of current knowledge, as these strains have DNA
165 sequences that correspond to *L. chinense* or *L. cavernicola* clades. Finally, the study also showed
166 that the DNA sequences of several strains did not correspond to any known *Leptobacillium* clade
167 and could therefore constitute new clades that have not been described to date.

168

169 **Taxonomy**

170

171 ***Leptobacillium cavernicola*** Leplat, *sp. nov.* MB844042 (Fig. 3)

172 *GenBank*: OM622527 (ITS), OM628786 (LSU), OM628847 (SSU), OM677786 (RPB1),
173 OM654326 (RPB2), OM654337 (TEF1- α)

174 *Systematic position*: Fungi, *Dikarya*, *Ascomycota*, *Pezizomycotina*, *Sordariomycetes*,
175 *Hypocreomycetidae*, *Hypocreales*, *Cordycipitaceae*

176 **Holotype**:—FRANCE. Prignac-et-Marcamps, 45° 2' 20" N, 0° 30' 6" W, 22 October 2018.
177 Isolated from surface sampling in the Pair-non-Pair cave, CBS 149113, preserved in a
178 metabolically inactive state.

179 *Etymology*: The epithet *cavernicola* refers to the environment from which the strains of the
180 species were mainly isolated, i.e. caves.

181 Colonies on MEA reached 12–24 mm diam. after 14 days at 25 °C, aerial mycelium can
182 be scarce, velvety, white, slightly yellow exudate present in some strains. Reverse dark brown.

183 Colonies on PDA reached 14–20 mm diam. after 14 days at 25 °C, aerial mycelium can
184 be scarce, velvety to floccose, white. Reverse usually dark brown. Yellow diffusible pigment
185 present in some strains.

186 Phialides 5.1–27.2 x 1.2–1.7 μm , mainly solitary, slender, tapering toward tip. Conidia 3.1–6.9
187 x 0.9–1.5 μm , forming long, slender chains, narrowly cylindrical to slightly fusiform, some were
188 slightly lemon-shaped, first-formed conidium were usually shorter, obovoid to pyriform with a
189 rounded distal end.

190 Chlamyospore absent. Octahedral crystals not observed.

191 Other strains examined: —FRANCE. LRMH C212, LRMH C216, LRMH C302, LRMH
192 C304, Cabrerets, 44° 30' 27" N, 1°38' 40" E, isolated from air samplings in Pech-Merle cave,
193 LRMH C212 and LRMH C216 in October 2016, LRMH C302 and LRMH C304 in October
194 2018; LRMH C217, Prignac-et-Marcamps, 45° 2' 20" N, 0° 30' 6" W, October 2016, isolated
195 from air sampling in Pair-non-Pair cave; LRMH C313, LRMH C314, Les Eyzies-de-Taillac-
196 Sireuil, 44° 56' 37" N, 1° 02' 32" E, October 2018, isolated from air samplings in Combarelles
197 cave.

198 *Notes: Leptobacillium cavernicola* is distinguishable in the genus *Leptobacillium* by its slow
199 growth rate on PDA at 25 °C, which is comparable to the rates recorded for *L. leptobactrum* var.
200 *leptobactrum* and *L. muralicola*. However, caution should be applied when comparing with *L.*
201 *muralicola* due to the differences in experimental protocol: Sun *et al.* (2019) studied the growth
202 rate of this fungus at 20 °C over 10 days. The phialides produced by *L. cavernicola* seem shorter
203 than those produced by *L. leptobactrum* var. *leptobactrum* and *L. muralicola*, and the difference
204 between these three species is significantly supported by the phylogenetic data. The dark brown
205 reverse of *L. cavernicola* on MEA and PDA media also seems to be characteristic of the species.

206

207

208

209

210 **Discussion**

211

212 The phylogenetic study associated with the morphological study of the fungal strains isolated
213 from a cave environment provides clear evidence that *Leptobacillium cavernicola* can be
214 described as a new species. The closest species was *L. muralicola*, both on morphological and
215 phylogenetic features. An interesting point is the presence of some similarities between the
216 isolation environment of *L. muralicola* and that of *L. cavernicola*. *Leptobacillium muralicola*
217 was isolated from mural paintings in a Koguryo tomb in China (Sun *et al.* 2019), while *L.*
218 *cavernicola* was isolated from Paleolithic decorated caves. Apart from the similar cultural value
219 of these sites, the two types of site are stone monuments that are usually subjected to high values
220 of relative humidity, as noted for another Koguryo tomb (Mazzeo *et al.* 2006). Indeed, the air in
221 caves is generally saturated with humidity (Bourges *et al.* 2014).

222 In general, the genus *Leptobacillium* seems to be relatively frequent in the cave environment,
223 since several *Leptobacillium* spp. were recovered in French, Spanish, German and Portuguese
224 caves (Martin-Sanchez *et al.* 2014; Dominguez-Moñino *et al.* 2021; Burow *et al.* 2019). The
225 authors generally linked the occurrence of *Leptobacillium* genus in caves with its entomogenous
226 characteristics, i.e. arthropods play a role in the dispersion of fungal strains in caves (Bastian *et*
227 *al.* 2009; Dominguez-Moñino *et al.* 2021). *Leptobacillium cavernicola* was not directly isolated
228 from arthropods in this study since only air and surface samplings were performed, but, likely,
229 this new species could also have similar entomogenous characteristics to those of other species
230 in the genus. It is also possible that the species has the capacity to survive and/or develop on
231 mineral substrate, as already noted for *L. leptobactrum* (Daghino *et al.* 2009).

232 It is interesting to note that *L. cavernicola* had probably already been detected in the past, but
233 had been identified as *L. leptobactrum* due to the similarity between the ITS sequences of several
234 strains and those determined for *L. cavernicola* sequences in this study. Several of these strains
235 were also recovered from a cave environment, including strains isolated from Cueva de Ardales

236 and Gruta de las Maravillas in Spain (Dominguez-Moñino *et al.* 2021), and from Lascaux cave
237 in France (Porca *et al.* 2011; Martin-Sanchez *et al.* 2014). In general, the taxonomic position of
238 many strains identified as *L. leptobactrum* should be reevaluated in the light of our findings.
239 While strains identified as *L. symbioticum* Okane, Nonaka, Kurihara, J.P. Abe & Yamaoka and
240 *L. coffeaneum* seem to form relatively consistent clades, many strains identified as *L.*
241 *leptobactrum* correspond to *L. chinense* or *L. cavernicola* clades in our study. Moreover, other
242 strains that did not correspond to *L. chinense* or *L. cavernicola* could actually be several new
243 species. Many of the currently misidentified strains were probably identified before the
244 description of the new species in the genus *Leptobacillum*. The description of the *Leptobacillum*
245 *leptobactrum* species type dates back to 2016 (Zare & Gams 2016), while the other species in
246 the genus had been described between 2019 and 2021 (Sun *et al.* 2019; Okane *et al.* 2020; Chen
247 *et al.* 2021). Another explanation could be that the misidentified strains mainly came from fungal
248 inventory studies that did not seek to describe new species, and these strains were therefore only
249 identified on one DNA locus alone, mainly the ITS region. The study of additional DNA loci
250 should facilitate a more precise identification of these existing strains, as reported in fungi in
251 general (Tekpinar & Kalmer 2019), and particularly in the Cordycipitaceae family (Kepler *et al.*
252 2017).

253 *Leptobacillum filiforme* is a more unusual case due to the limited amount of data (available for
254 only one strain). The phylogenetic position of this species appears to be very close to *L.*
255 *symbioticum*. The size of the conidia in *L. filiforme* species seems larger than in *L. symbioticum*
256 species (Okane *et al.* 2020; Crous *et al.* 2018), a finding that justifies the existence of two distinct
257 species.

258

259 **Acknowledgments**

260 We thank Joanna Lignot for English language editing.

261 The OPerCul workstation used to perform the robustness calculations for the phylogenetic
262 studied was supported by the Paris Ile-de-France Region – DIM “Matériaux anciens et
263 patrimoniaux”.

264

265 **References**

266 Bastian F., Alabouvette C. & Saiz-Jimenez C. (2009) The impact of arthropods on fungal
267 community structure in Lascaux Cave. *Journal of Applied Microbiology* 106 (5): 1456-
268 1462. DOI: 10.1111/j.1365-2672.2008.04121.x

269 Bourges F., Genthon P., Genty D., Lorblanchet M., Mauduit E. & D'Hulst D. (2014)
270 Conservation of prehistoric caves and stability of their inner climate: lessons from
271 Chauvet and other French caves. *Science of the Total Environment* 493: 79-91. DOI:
272 10.1016/j.scitotenv.2014.05.137

273 Burow K., Grawunder A., Harpke M., Pietschmann S., Ehrhardt R., Wagner L., Voigt K., Merten
274 D., Büchel G. & Kothe E. (2019) Microbiomes in an acidic rock–water cave system.
275 *FEMS Microbiology Letters* 366 (13): 1-11. DOI: 10.1093/femsle/fnz167

276 Castlebury L.A., Rossmann A.Y., Gi-Ho S., Hyten A.S. & Spatafora J.W. (2004) Multigene
277 phylogeny reveals new lineage for *Stachybotrys chartarum*, the indoor air fungus.
278 *Mycological Research* 108 (8): 864-872. DOI: 10.1017/S0953756204000607

279 Chen W.-H., Han Y.-F., Liang J.-D. & Liang Z.-Q. (2021) Taxonomic and phylogenetic
280 characterizations reveal four new species of *Simplicillium* (Cordycipitaceae,
281 Hypocreales) from Guizhou, China. *Scientific Reports* 11 (1): 1-12. DOI:
282 10.1038/s41598-021-94893-z

- 283 Chen W.-H., Liu C., Han Y.-F., Liang J.-D., Tian W.-Y. & Liang Z.-Q. (2019) Three novel
284 insect-associated species of *Simplicillium* (Cordycipitaceae, Hypocreales) from
285 Southwest China. *MycKeys* 58: 83-102. DOI: 10.3897/mycokeys.58.37176
- 286 Clark K., Karsch-Mizrachi I., Lipman D.J., Ostell J. & Sayers E.W. (2016) GenBank. *Nucleic
287 Acids Research* 44 (D1): D67-D72. DOI: 10.1093/nar/gkv1276
- 288 Crous P., Luangsa-Ard J., Wingfield M., Carnegie A., Hernández-Restrepo M., Lombard L.,
289 Roux J., Barreto R., Baseia I. & Cano-Lira J. (2018) Fungal Planet description sheets:
290 785–867. *Persoonia: Molecular Phylogeny and Evolution of Fungi* 41: 238-417. DOI:
291 10.3767/persoonia.2018.41.12
- 292 Crous P.W., Gams W., Stalpers J.A., Robert V. & Stegehuis G. (2004) MycoBank: an online
293 initiative to launch mycology into the 21st century. *Studies in Mycology* 50 (1): 19-22.
- 294 Daghino S., Turci F., Tomatis M., Girlanda M., Fubini B. & Perotto S. (2009) Weathering of
295 chrysotile asbestos by the serpentine rock-inhabiting fungus *Verticillium leptobactrum*.
296 *FEMS microbiology ecology* 69 (1): 132-141. DOI: 10.1111/j.1574-6941.2009.00695.x
- 297 Dominguez-Moñino I., Jurado V., Rogerio-Candelera M.A., Hermosin B. & Saiz-Jimenez C.
298 (2021) Airborne fungi in show caves from southern Spain. *Applied Sciences* 11 (11):
299 5027. DOI: 10.3390/app11115027
- 300 Edel V., Steinberg C., Gautheron N., Recorbet G. & Alabouvette C. (2001) Genetic diversity of
301 *Fusarium oxysporum* populations isolated from different soils in France. *FEMS
302 Microbiology Ecology* 36: 61-71. DOI: 10.1016/S0168-6496(01)00119-2
- 303 Edler D., Klein J., Antonelli A. & Silvestro D. (2019) raxmlGUI 2.0 beta: a graphical interface
304 and toolkit for phylogenetic analyses using RAxML. *BioRxiv*: 800912. DOI:
305 10.1101/800912
- 306 Gams W. (1988) A contribution to the knowledge of nematophagous species of *Verticillium*.
307 *Netherlands Journal of Plant Pathology* 94 (3): 123-148. DOI: 10.1007/BF01978003

308 Gardes M. & Bruns T.D. (1993) ITS primers with enhanced specificity for basidiomycetes-
309 application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2 (2): 113-
310 118. DOI: 10.1111/j.1365-294X.1993.tb00005.x

311 Gomes A.A., Pinho D.B., Cardeal Z., Menezes H.C., De Queiroz M.V. & Pereira O.L. (2018)
312 *Simplicillium coffeanum*, a new endophytic species from Brazilian coffee plants, emitting
313 antimicrobial volatiles. *Phytotaxa* 333 (2): 188-198. DOI: 10.11646/phytotaxa.333.2.2

314 Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis
315 program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41 (41): 95-98.

316 Katoh K., Rozewicki J. & Yamada K.D. (2019) MAFFT online service: multiple sequence
317 alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* 20
318 (4): 1160-1166. DOI: 10.1093/bib/bbx108

319 Kepler R.M., Luangsa-Ard J.J., Hywel-Jones N.L., Quandt C.A., Sung G.-H., Rehner S.A., Aime
320 M.C., Henkel T.W., Sanjuan T. & Zare R. (2017) A phylogenetically-based nomenclature
321 for Cordycipitaceae (Hypocreales). *IMA fungus* 8 (2): 335-353. DOI:
322 10.5598/imafungus.2017.08.02.08

323 Kumar S., Stecher G., Li M., Knyaz C. & Tamura K. (2018) MEGA X: molecular evolutionary
324 genetics analysis across computing platforms. *Molecular Biology and Evolution* 35 (6):
325 1547-1549. DOI: 10.1093/molbev/msy096

326 Lemoine F., Correia D., Lefort V., Doppelt-Azeroual O., Mareuil F., Cohen-Boulakia S. &
327 Gascuel O. (2019) NGPhylogeny.fr: new generation phylogenetic services for non-
328 specialists. *Nucleic Acids Research* 47 (W1): W260-W265. DOI: 10.1093/nar/gkz303

329 Liu F. & Cai L. (2012) Morphological and molecular characterization of a novel species of
330 *Simplicillium* from China. *Cryptogamie, Mycologie* 33 (2): 137-144. DOI:
331 10.7872/crym.v33.iss2.2012.137

- 332 Liu Y.J., Whelen S. & Hall B.D. (1999) Phylogenetic relationships among ascomycetes:
333 evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* 16 (12):
334 1799-1808. DOI: 10.1093/oxfordjournals.molbev.a026092
- 335 Martin-Sanchez P.M., Jurado V., Porca E., Bastian F., Lacanette D., Alabouvette C. & Saiz-
336 Jimenez C. (2014) Airborne microorganisms in Lascaux Cave (France). *International*
337 *Journal of Speleology* 43 (3): 295-303. DOI: 10.5038/1827-806X.43.3.6
- 338 Matheny P.B., Liu Y.J., Ammirati J.F. & Hall B.D. (2002) Using RPB1 sequences to improve
339 phylogenetic inference among mushrooms (Inocybe, Agaricales). *American Journal of*
340 *Botany* 89 (4): 688-698. DOI: 10.3732/ajb.89.4.688
- 341 Mazzeo R., Joseph E., Minguzzi V., Grillini G., Baraldi P. & Prandstraller D. (2006) Scientific
342 investigations of the Tokhung-Ri tomb mural paintings (408 AD) of the Koguryo era,
343 Democratic People's Republic of Korea. *Journal of Raman Spectroscopy* 37 (10): 1086-
344 1097. DOI: 10.1002/jrs.1592
- 345 Mongkolsamrit S., Noisripoom W., Thanakitpipattana D., Wutikhun T., Spatafora J.W. &
346 Luangsa-Ard J. (2018) Disentangling cryptic species with isaria-like morphs in
347 Cordycipitaceae. *Mycologia* 110 (1): 230-257. DOI: 10.1080/00275514.2018.1446651
- 348 Okane I., Nonaka K., Kurihara Y., Abe J.P. & Yamaoka Y. (2020) A new species of
349 *Leptobacillium*, *L. symbioticum*, isolated from mites and sori of soybean rust. *Mycoscience*
350 61 (4): 165-171. DOI: 10.1016/j.myc.2020.04.006
- 351 Porca E., Jurado V., Martin-Sanchez P.M., Hermosin B., Bastian F., Alabouvette C. & Saiz-
352 Jimenez C. (2011) Aerobiology: An ecological indicator for early detection and control
353 of fungal outbreaks in caves. *Ecological Indicators* 11: 1594-1598. DOI:
354 10.1016/j.ecolind.2011.04.003

- 355 Regaieg H., Ciancio A., Raouani N.H. & Rosso L. (2011) Detection and biocontrol potential of
356 *Verticillium leptobactrum* parasitizing *Meloidogyne* spp. *World Journal of Microbiology*
357 *and Biotechnology* 27 (7): 1615-1623. DOI: 10.1007/s11274-010-0615-0
- 358 Rehner S. & Buckley E. (2005) A *Beauveria* phylogeny inferred from nuclear ITS and EF1-a
359 sequences: evidence for cryptic diversification and links to *Cordyceps* teleomorphs.
360 *Mycologia* 97 (1): 84-98. DOI: 10.1080/15572536.2006.11832842
- 361 Ronquist F., Teslenko M., Van Der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu
362 L., Suchard M.A. & Huelsenbeck J.P. (2012) MrBayes 3.2: efficient Bayesian
363 phylogenetic inference and model choice across a large model space. *Systematic Biology*
364 61 (3): 539-542. DOI: 10.1093/sysbio/sys029
- 365 Stamatakis A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of
366 large phylogenies. *Bioinformatics* 30 (9): 1312-1313. DOI:
367 10.1093/bioinformatics/btu033
- 368 Sun J.-Z., Ge Q.-Y., Zhu Z.-B., Zhang X.-L. & Liu X.-Z. (2019) Three dominating hypocrealean
369 fungi of the 'white mold spots' on acrylic varnish coatings of the murals in a Koguryo
370 tomb in China. *Phytotaxa* 397 (3): 225-236. DOI: 10.11646/phytotaxa.397.3
- 371 Talavera G. & Castresana J. (2007) Improvement of phylogenies after removing divergent and
372 ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56
373 (4): 564-577. DOI: 10.1080/10635150701472164
- 374 Tekpinar A.D. & Kalmer A. (2019) Utility of various molecular markers in fungal identification
375 and phylogeny. *Nova Hedwigia* 109 (1-2): 187-224. DOI:
376 10.1127/nova_hedwigia/2019/0528
- 377 Vilgalys R. & Hester M. (1990) Rapid genetic identification and mapping of enzymatically
378 amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*
379 172 (8): 4238-4246. DOI: 10.1128/jb.172.8.4238-4246

- 380 Wang Y.-B., Wang Y., Fan Q., Duan D.-E., Zhang G.-D., Dai R.-Q., Dai Y.-D., Zeng W.-B.,
381 Chen Z.-H. & Li D.-D. (2020) Multigene phylogeny of the family Cordycipitaceae
382 (Hypocreales): new taxa and the new systematic position of the Chinese cordycipitoid
383 fungus *Paecilomyces hepiali*. *Fungal Diversity*: 1-46. DOI: 10.1007/s13225-020-00457-
384 3
- 385 White T.J., Bruns T., Lee S.J.W.T. & Taylor J.W. (1990) *Amplification and direct sequencing of*
386 *fungus Paecilomyces hepiali*. *Fungal Diversity*: 1-46. DOI: 10.1007/s13225-020-00457-
387 White TJ (eds) *PCR protocols: a guide to methods and applications*. vol 1. Academic
388 Press, Inc, San Diego (USA), pp 315-322
- 389 Zare R. & Gams W. (2001) A revision of *Verticillium* section *Prostrata*. IV. The genera
390 *Lecanicillium* and *Simplicillium* gen. nov. *Nova Hedwigia* 73 (1): 1-50. DOI:
391 10.1127/nova.hedwigia/71/2001/1
- 392 Zare R. & Gams W. (2016) More white verticillium-like anamorphs with erect conidiophores.
393 *Mycological Progress* 15 (10-11): 993-1030. DOI: 10.1007/s11557-016-1214-8
- 394 Zhang D., Gao F., Jakovlić I., Zou H., Zhang J., Li W.X. & Wang G.T. (2020a) PhyloSuite: an
395 integrated and scalable desktop platform for streamlined molecular sequence data
396 management and evolutionary phylogenetics studies. *Molecular Ecology Resources* 20
397 (1): 348-355. DOI: 10.1111/1755-0998.13096
- 398 Zhang Z.-F., Zhou S.-Y., Eurwilaichitr L., Ingsriswang S., Raza M., Chen Q., Zhao P., Liu F. &
399 Cai L. (2020b) Culturable mycobiota from Karst caves in China II, with descriptions of
400 33 new species. *Fungal Diversity*: In press. DOI: 10.1007/s13225-020-00453-7

401

402 **TABLE 1.** Strains of *Leptobacillum cavernicola* studied

Collection number	Sampling place	Sampling date	Sampling method
LRMH C212	Pech-Merle cave (Occitanie, France)	10/2016	Air sampling
LRMH C216	Pech-Merle cave (Occitanie, France)	10/2016	Air sampling
LRMH C217	Pair-non-Pair cave (Nouvelle-Aquitaine, France)	10/2016	Air sampling
LRMH C248*	Pair-non-Pair cave (Nouvelle-Aquitaine, France)	10/2016	Air sampling
LRMH C251*	Font-de-Gaume cave (Nouvelle-Aquitaine, France)	10/2016	Air sampling
LRMH C299	Pair-non-Pair cave (Nouvelle-Aquitaine, France)	10/2018	Surface sampling
LRMH C302	Pech-Merle cave (Occitanie, France)	10/2018	Air sampling
LRMH C304	Pech-Merle cave (Occitanie, France)	10/2018	Air sampling
LRMH C312*	Combarelles cave (Nouvelle-Aquitaine, France)	10/2018	Air sampling
LRMH C313	Combarelles cave (Nouvelle-Aquitaine, France)	10/2018	Air sampling
LRMH C314	Combarelles cave (Nouvelle-Aquitaine, France)	10/2018	Air sampling

403 * Strains included in the phylogenetic study alone; the preserved strains did not grow back from the collection tubes and were therefore
 404 excluded

Accepted Version

405 **TABLE 2.** Fungal taxa used in the phylogenetic study of *Leptobacillium cavernicolum*.

Species	Collection number	ITS	SSU	LSU	TEF1- α	RPB1	RPB2	Reference study regarding the phylogenetic placement
<i>Leptobacillium cavernicola</i>	LRMH C212	OM622523	OM628842	OM628781	OM654332	OM677781	OM654321	This study
<i>L. cavernicola</i>	LRMH C216	OM622524	OM628843	OM628782	OM654333	OM677782	OM654322	This study
<i>L. cavernicola</i>	LRMH C217	MF788211	OM628844	OM628783	OM654334	OM677783	OM654323	This study
<i>L. cavernicola</i>	LRMH C248	OM622525	OM628845	OM628784	OM654335	OM677784	OM654324	This study
<i>L. cavernicola</i>	LRMH C251	OM622526	OM628846	OM628785	OM654336	OM677785	OM654325	This study
<i>L. cavernicola</i>	LRMH C299, CBS 149113	OM622527	OM628847	OM628786	OM654337	OM677786	OM654326	This study
<i>L. cavernicola</i>	LRMH C302	OM622528	OM628848	OM628787	OM654338	OM677787	OM654327	This study
<i>L. cavernicola</i>	LRMH C304	OM622529	OM628849	OM628788	OM654339	OM677788	OM654328	This study
<i>L. cavernicola</i>	LRMH C312	OM622530	OM628850	OM628789	OM654340	OM677789	OM654329	This study
<i>L. cavernicola</i>	LRMH C313	OM622531	OM628851	OM628790	OM654341	OM677790	OM654330	This study
<i>L. cavernicola</i>	LRMH C314	OM622532	OM628852	OM628791	OM654342	OM677791	OM654331	This study
<i>L. chinense</i>	CGMCC 3.14969	JQ410323	(-)	JQ410321	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>L. chinense</i>	CGMCC 3.14970	JQ410324	(-)	JQ410322	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>L. chinense</i>	NTUCC 20-073	MT974199	(-)	MT974414	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>L. coffeanum</i>	COAD 2057	MF066034	(-)	MF066032	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>L. coffeanum</i>	COAD 2061	MF066035	(-)	MF066033	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>L. filiforme</i>	URM 7918	MH979338	(-)	MH979399	(-)	(-)	(-)	(Chen <i>et al.</i> 2021)
<i>L. leptobactrum</i> var. <i>calidius</i>	CBS 703.86	EF641866	EF641850	KU382226	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>calidius</i>	CBS 748.73	EF641867	EF641851	KU382227	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>calidius</i>	NFCCI 4235	MG786580	(-)	MG786581	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>leptobactrum</i>	CBS 414.70	MH859773	EF641846	MH871535	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>leptobactrum</i>	CBS 771.69	EF641868	EF641852	KU382224	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>leptobactrum</i>	CBS 774.69	MH859421	(-)	MH871192	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>leptobactrum</i>	CBS 775.69	MH859422	(-)	MH871193	(-)	(-)	(-)	(Zare & Gams 2016)
<i>L. leptobactrum</i> var. <i>leptobactrum</i>	JCM 39056	LC496868	LC496903	LC496888	LC496918	(-)	(-)	(Zare & Gams 2016)
<i>L. muralicola</i>	CGMCC 3.19014	MH379983	(-)	MH379997	(-)	(-)	(-)	(Sun <i>et al.</i> 2019)
<i>L. muralicola</i>	CGMCC 3.19016	MH379986	(-)	MH380000	(-)	(-)	(-)	(Sun <i>et al.</i> 2019)
<i>L. muralicola</i>	CGMCC 3.19015	MH379985	(-)	MH379999	(-)	(-)	(-)	(Sun <i>et al.</i> 2019)
<i>L. symbioticum</i>	NBRC 104297	LC485674	(-)	AB378539	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>L. symbioticum</i>	NBRC 113865	LC485673	(-)	LC506046	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)

<i>L. symbioticum</i>	OPTF00168	LC485675	(-)	LC506047	(-)	(-)	(-)	(Okane <i>et al.</i> 2020)
<i>Gamszarea wallacei</i>	CBS 101237	EF641891	AY184978	AY184967	EF469073	EF469102	EF469119	(Zhang <i>et al.</i> 2020b)

406

Accepted Version

407 **TABLE 3.** Primers used to amplify *Leptobacillum cavernicola* strains in this study.

Target gene	Primer	Primer DNA sequence	Annealing temperature	Reference
ITS	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	50°C	(Gardes & Bruns 1993)
	ITS4	TCCTCCGCTTATGATATGC		(White <i>et al.</i> 1990)
LSU	LR0R	GTACCCGCTGAACTTAAGC	56 °C	(Vilgalys & Hester 1990)
	LR5	ATCCTGAGGGAAACTTC		
SSU	NS1	GTAGTCATATGCTTGCTC	55 °C	(White <i>et al.</i> 1990)
	NS4	CTCCGTC AATTCCTTTAAG		
TEF1- α	983F	GCYCCYGGHCA YCGTGAYTTYAT	55 °C	(Rehner & Buckley 2005)
	2218R	ATGACACCRACRGC RACRGTYTG		
RPB1	CRPB1	CCWGGYTTYATCAAGAARGT	55 °C	(Castlebury <i>et al.</i> 2004)
	RPB1Cr	CCNGCDATNCRTRTRCCATRTA		(Matheny <i>et al.</i> 2002)
RPB2	fRPB2-5f	GAYGAYMGWGATCAYTTYGG	58 °C	(Liu <i>et al.</i> 1999)
	fRPB2-7cR	CCCATRGCTTGYTTRCCCAT		

408

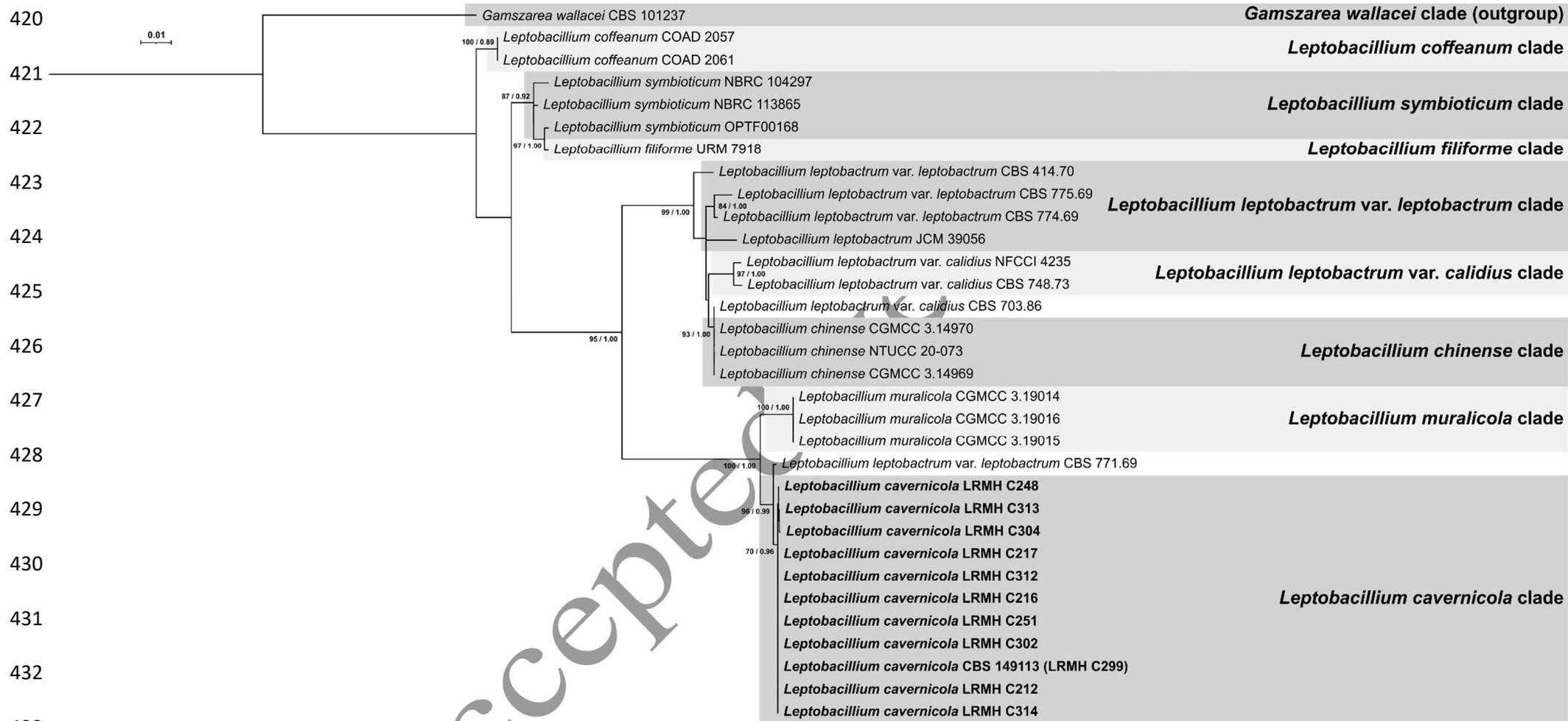
Accepted Version

409 **Figure 1.** Phylogenetic studies of *Leptobacillium cavernicola* strains through maximum
410 likelihood and Bayesian inference in relation to *Leptobacillium* spp. strains for which at least
411 two different DNA loci were available in the NCBI GenBank database.

412 The trees were obtained from a dataset including 32 taxa leading to a concatenated alignment
413 of 4311 bp from six nuclear regions (ITS, LSU, SSU, RPB1, RPB2 and TEF1- α). The trees
414 were rooted with *Gamszarea wallacei* CBS 101237 as an outgroup. Bootstrap values greater
415 than or equal to 70 % and Bayesian posterior probabilities greater than or equal to 0.70 are
416 located close to the corresponding node. The specific strains studied are printed in bold. The
417 two strains that are not included in a gray area are those for which the identification is uncertain
418 according to the phylogenetic studies.

419

Accepted Version

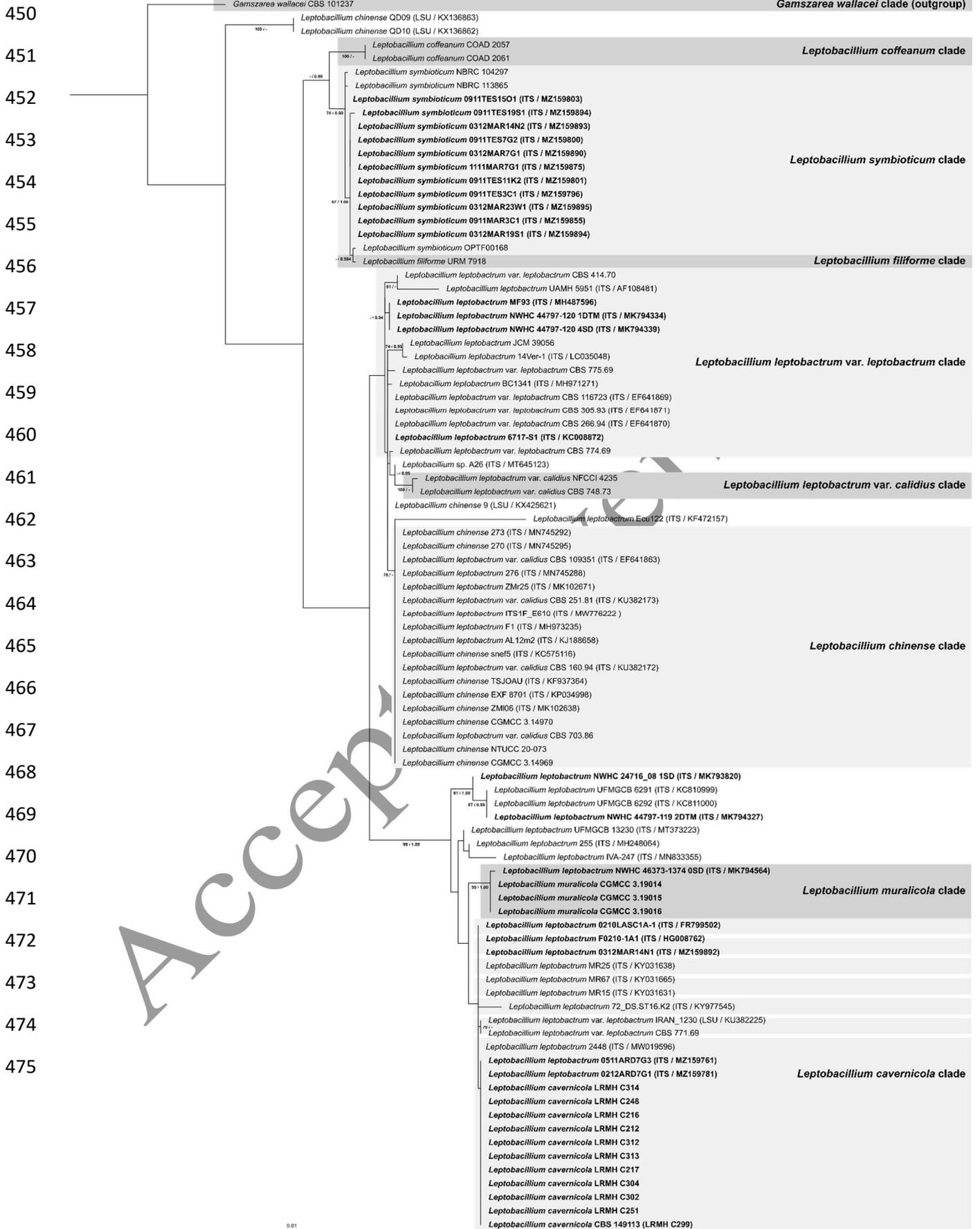


Accepted Article

435 **FIGURE 2.** Phylogenetic studies of *Leptobacillium cavernicola* strains through maximum
436 likelihood and Bayesian inference in relation to all *Leptobacillium spp.* strains available in the
437 NCBI GenBank database.

438 The trees were obtained from a dataset including 91 taxa leading to a concatenated alignment
439 of 1074 bp from two nuclear regions (ITS, LSU). The trees were rooted with *Gamszarea*
440 *wallacei* CBS 101237 as an outgroup. Bootstrap values greater than or equal to 70 % and
441 Bayesian posterior probabilities greater than or equal to 0.70 are located close to the
442 corresponding node. The DNA locus sequenced and the accession number for each strain are
443 indicated in parentheses. The strains without details of DNA locus and accession number are
444 those already used in the first phylogenetic study. The strains in bold print correspond to strains
445 isolated from hypogean environments in general, and caves in particular. The strains that are
446 not included in a gray area are those that cannot be clearly sorted within an existing
447 *Leptobacillium* clade. Strains in the hatched gray area probably belong to *Leptobacillium*
448 *cavernicola* clade.

449



476 **FIGURE 3.** *Leptobacillium cavernicola* (Photographer: J. LEPLAT)
477 a–b. Upper and reverse views of the culture on MEA (14 d at 25 °C). c–d. Upper and reverse
478 views of the culture on PDA (14 d at 25 °C). e. Long chains of cylindrical to fusiform
479 microconidia. The arrow indicates the typical first-formed conidium, which is shorter, obovoid
480 to pyriform with a rounded distal end. f. Phialides, indicated by arrows. Scale bars: e–f = 10
481 μm .

Accepted Version

482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506

