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# The diversity of mycolactone-producing mycobacteria

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**Keywords:** *Mycobacterium*, *Mycobacterium ulcerans*, taxonomy, diversity, clones, *Mycobacterium marinum*, mapping.

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23 **ABSTRACT**

24 Mycolactone-producing mycobacteria (MPM) form an intriguing group of  
25 environmental opportunistic pathogens of mammals and human patients in whom  
26 they cause cutaneous and subcutaneous ulcers known as “Buruli ulcer” when they  
27 occur in humans. We reviewed whole genome sequence data and ecological and  
28 phenotypic characteristics from 44 MPMs and closely related *Mycobacterium*  
29 *marinum*. This analysis indicated that all the 24 *M. marinum* isolates were delineated  
30 into seven taxa and our comprehensive, polyphasic taxonomic approach led to the  
31 proposal of delineating *M. marinum* genomospecies, 01-07. Likewise, 20 MPMs  
32 isolates were delineated into seven additional *M. ulcerans* genomospecies, 01-07. A  
33 taxonomic card explaining the ecology, hosts of isolation and the plasmid harboured  
34 is provided for each taxon.

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## 39 INTRODUCTION

40 An unique group of opportunistic mycobacterial pathogens secreting plasmid-  
41 encoded mycolactone exotoxins known as mycolactone-producing-mycobacteria  
42 (MPM), is comprised of mycobacteria currently classified as *Mycobacterium marinum*  
43 (*M. marinum*) and *Mycobacterium ulcerans* (*M. ulcerans*) [1] MPMs are unique, being  
44 the sole mycobacteria known to secrete toxins and are among the mycobacteria that  
45 exhibit an extrachromosomal plasmid [2]. Mycolactone is a cytotoxin molecule that  
46 causes defective cell adhesion and directional migration of epithelial cells as a result  
47 of altered actin assembly and distribution in the cytoplasm by diverting the Wiskott-  
48 Aldrich Syndrome Protein (WASP) family of proteins [3]. In addition, mycolactone  
49 exhibits analgesic properties and immunomodulates the inflammatory responses of  
50 sensory neurons, Schwann cells and microglia [4], and induces keratinocyte cell  
51 necrosis causing cutaneous and subcutaneous ulcers in mammals, including in  
52 human patients in whom such lesions are known as Buruli ulcer [5]. However, these  
53 mycolactone properties may be as opportunistic as the pathogen itself and we  
54 recently reported that one primal function of mycolactones was to make immotile *M.*  
55 *ulcerans* communicate with some fungi, attracting those fungi thriving in shared  
56 ecological niches [6].

57 *M. marinum* was first isolated in 1926 by Aronson [7], and was recognised as  
58 an opportunistic pathogen for humans in 1951, after it had been isolated from  
59 granulomatous skin lesions in swimmers [8]. The clinical manifestation of infections in  
60 humans consists of nodular skin lesions that can progress to tenosynovitis, arthritis  
61 and osteomyelitis [9]. We also described vivarium granuloma as the *M. marinum*  
62 cutaneous infection following contact with reptiles [10]. As for Buruli ulcer, it is a  
63 World Health Organization (WHO) notifiable infection and is acknowledged as the

64 third most common mycobacterial disease after tuberculosis and leprosy in  
65 populations in several west and central African countries [11]. The current dynamics  
66 of Buruli ulcer reveals a global decrease in prevalence and incidence in 2010–2017,  
67 with a drastic decrease in Côte d'Ivoire, which was once the country reporting the  
68 largest number of cases, while the proportion of cases reported in Australia is  
69 increasing, as it is in other countries such as Ghana and Nigeria [12,13]. Also, a few  
70 cases have been reported in Senegal, a country recently regarded as free of Buruli  
71 ulcer [14]. In Australia, not only is Buruli ulcer reemerging in south-eastern regions and  
72 spreading to regions that were previously free of Buruli ulcer, but an increasing  
73 number of severe cases is bringing into question the diversity of the strains that  
74 cause *M. ulcerans* [12].

75 MPMs are acknowledged as environmental mycobacteria, yet the exact  
76 ecosystems in which they thrive in tropical regions which are endemic for Buruli  
77 ulcer, remain elusive mainly because past investigations were conducted using  
78 PCR-based methods rather than culture, leaving the exact nature and viability of  
79 detected mycobacteria unclear. As for *M. ulcerans*, the significant association of  
80 Buruli ulcer cases with wetlands and overflowing riverbanks and the detection of  
81 specific sequences of *M. ulcerans* in water, mud, aquatic insects, and plants has  
82 suggested that *M. ulcerans* lives in fresh water-irrigated environments [15]. However,  
83 the mode(s) of transmission, natural reservoir(s) and other key aspects of the  
84 epidemiology of Buruli ulcer are not fully understood [16], despite *M. ulcerans* being  
85 recently traced along a food chain, in Côte d'Ivoire [Hammoudi N., Drancourt M,  
86 unpublished data]. The paradoxical situation for environmental opportunistic  
87 pathogens, where fewer environmental than clinical isolates hampers any rational  
88 prevention of the disease which, although not contagious and not responsible for any

89 mortality, nevertheless has a huge medical and social impact on populations and is  
90 responsible for functional limitations that have an impact on the social life of affected  
91 individuals.

92 Initial whole genome sequence comparisons of *M. ulcerans* and *M. marinum*  
93 all indicate a reductive evolution from a common ancestor closely related to *M.*  
94 *marinum*, counterbalanced by lateral gene transfer, the acquisition of insertion  
95 sequences named IS2404 and IS2606, and the acquisition of a 174-kb plasmid  
96 encoding gene for the non-ribosomal synthesis of polyketide mycolactones [17-19].  
97 Further, PCR-restriction profile analyses of the 16S rRNA gene separated four  
98 different genotypic profiles (African, South-East Asian, Mexican and South American)  
99 in *M. ulcerans* [20], while nine distinct profiles identified by PCR 2426 correlated with  
100 the geographical origin of the isolates [21]. Analysis of specific deletions in the  
101 Regions of Difference (RD) revealed that *M. ulcerans* evolved into five InDel  
102 haplotypes that separated *M. ulcerans* isolates into so-called “classic” and “ancestral”  
103 lineages [22]. A genomic analysis carried out on 123 genomes from the territory of  
104 Songololo in the Democratic Republic of Congo (DRC), revealed that several *M.*  
105 *ulcerans* clones coexist and circulate simultaneously in this region with a  
106 homogeneous geographical distribution [23]. However, these genetic and genome  
107 sequence-based analyses did not consider the ecology and biology of the isolates.

108 In this study, by combining the ever larger collection of whole genome  
109 sequences with ecological and phenotypic data, we propose an updated taxonomy  
110 based on the polyphasic deviation of seven taxa, for which a nomenclature is  
111 proposed.

112 **GENOMIC DATA.**

113 **MPM genomic general data.** A total of 44 whole genome sequences (annotated as  
114 *M. marinum* (n = 33), *M. ulcerans* (n = 7), *Mycobacterium liflandii* (n = 1),  
115 *Mycobacterium pseudoshottsii* (n = 2) and one unnamed *Mycobacterium sp.* 012931  
116 were retrieved from the Pubmed assembly databases using *M. marinum* and MPM  
117 as keywords. Taken together, these genomes, consisting of 260 million bases,  
118 exhibited a GC% varying between 62.3% for *M. ulcerans subsp. shinshuensis* strain  
119 ATCC 33728 (AP017635.1), 63.4% for *M. ulcerans* CSURP9951 and 65.2-65.7% for  
120 the 42 remaining isolates (Supplementary Fig. S1).

121

122 **Whole genome sequence analysis.** Average nucleotide identity (ANI) analysis  
123 revealed using MORPHEUS (Versatile matrix visualisation and analysis software)  
124 delineated two large clusters and several small clusters, the *M. marinum* group (1)  
125 and the MPMs group (2) (Fig. 1). In detail, on the *M. marinum* group (1) we identified  
126 seven different taxa, namely taxon (1.1) including 16 *M. marinum* isolates exhibiting  
127 99.32-99.91% ANI values (Fig 1). While eight additional *M. marinum* isolates formed  
128 six small groups with ANI values of 97-98.8% with taxon (1.1). In particular, the *M.*  
129 *marinum* strain Davis1 forms a taxon (1.2) and exhibits ANI values of 98.14-98.88%  
130 with *M. marinum* taxon (1.1). *M. marinum* B and *M. marinum* MB2 grouped together  
131 and formed a taxon (1.3) with 99.84% identity, and *M. marinum* M and *M. marinum*  
132 E11 grouped together to form a taxon (1.4) with 99.88% identity, while *M. marinum*  
133 SS2 (1.5), *M. marinum* L1506 (1.6) and *M. marinum* DE 4576 (1.7) stood alone and  
134 formed three distinct taxa. Taken together, the ANI data indicated that isolates  
135 currently classified as *M. marinum* consist of new different taxa.

136           Regarding the MPMs isolates, we observed that the taxon (2) (Fig 1), was  
137 divided into five large groups One taxon (2.1) represented by six *M. ulcerans* namely  
138 *M. ulcerans subsp. shinshuense* ATCC 33728 (AP017635.1), *M. ulcerans subsp.*  
139 *shinshuense* ATCC33728 (AP017624.1), *M. ulcerans* CSURP9951, *M. ulcerans*  
140 CSURP9953, *M. ulcerans* CSURP9954 and *M. ulcerans* CSURP9952 isolates was  
141 supported by ANI values of 99.88- 99.93%. One taxon (2.2) containing *M. ulcerans*  
142 S4018, *M. ulcerans* SGL03, *M. ulcerans* Agy99, *M. ulcerans* CSURQ0185, *M.*  
143 *ulcerans* Harvey, *M. ulcerans* CSURP9950 and *M. ulcerans* CSURP9949 (99.57-  
144 99.92%); and *M. ulcerans* CSURP7741 stood alone and formed a taxon (2.3) with  
145 99.37% with taxon (2.4), which consisted of *M. liflandii*128FXT and *M. marinum*  
146 BB170200 with ANI value of 99.61%. One taxon (2.5) comprised *Mycobacterium sp.*  
147 012931, *M. marinum* DL240490, *M. pseudoshottsi* JCM 15466 and *M. pseudoshottsi*  
148 L15 with ANI values of 99.69-99.87%. Further WGS-based DDH analysis of 44  
149 isolates using formula 2 [24] yielded inconsistent results, and we observed that there  
150 were inconsistent results between the three DDH formula for the isolates studied  
151 (Fig. S2).

152

153 **Core genomics.** These 44 isolates yielded a 18,592-gene pangenome, a 2,250-  
154 gene, 2,104,697-bp core genome and 16,342 accessory genes which were  
155 accessory genes composed of 11,535 cloud genes (0% <= strains < 15%), 4,065  
156 shell genes (15% <= strains < 95%) and 742 soft core genes (95% <= strains <  
157 99%). A core-genome tree confirmed the ANI results with one large *M. marinum*  
158 taxon (1.1) including 16 *M. marinum* isolates and six discrete *M. marinum* taxa (1.2)  
159 (1.3) (1.4) (1.5) (1.6) and (1.7). Regarding MPMs, a core-genome tree confirmed the  
160 ANI results of a 5-taxon tree, but we demonstrated that the taxon (2.2) containing

161 seven *M. ulcerans* isolates was subdivided into three taxa, including taxon (2.2.1)  
162 formed by *M. ulcerans* CSURP9949, *M. ulcerans* CSURP9950 and *M. ulcerans* str.  
163 Harvey; taxon (2.2.2) including *M. ulcerans* Agy99, *M. ulcerans* SGL 03 and *M.*  
164 *ulcerans* 4018; and taxon (2.2.3) represented by *M. ulcerans* CSURQ0185 (Fig. 2).

165

166 **SNP-core genome analysis.** A pairwise SNP genetic distance tree of the 44 studied  
167 isolates confirmed the ANI and core-genome tree separating the MPMs from *M.*  
168 *marinum* (Fig. 3). Regarding the *M. marinum* taxon (1), we observed a group (1.1)  
169 including 16 *M. marinum* isolates with cut-off SNP varying between 1 and 5,604;  
170 taxon (1.2) related to taxon (1.1) of *M. marinum* taxon with SNP values varying  
171 between 13,841 and 14,670; taxa (1.3) (1.4) (1.5) (1.6) (1.7) far from taxon (1.1) with  
172 SNP values over 21,000. In detail, taxon (1.3) including two strains with 152-SNP  
173 values, and the group (1.4) including two strains with 0-SNP values. Regarding the  
174 MPMs taxon (2), we found that the taxon (2.1) including *M. ulcerans* subsp.  
175 *shinshuense* ATCC 33728 (AP017635.1), *M. ulcerans* subsp. *shinshuense*  
176 ATCC33728 (AP017624.1), *M. ulcerans* CSURP9951, *M. ulcerans* CSURP9953, *M.*  
177 *ulcerans* CSURP9954 and *M. ulcerans* CSURP9952 grouped together with SNP  
178 pairwise distance varying between 7-198 SNP; taxon (2.2.1) including *M. ulcerans*  
179 CSURP9949, *M. ulcerans* CSURP9950 and *M. ulcerans* str. Harvey with SNP 32-  
180 206; taxon (2.2.2) *M. ulcerans* Agy99, *M. ulcerans* strain SGL03 and *M. ulcerans*  
181 4018 with SNP 70-84; taxon (2.2.3) including only *M. ulcerans* CSURQ0185 close to  
182 taxon (2.2.1) with SNP 1526-1669 and taxon (2.2.2) with SNP 1973-1991. Taxon  
183 (2.3) including *M. ulcerans* CSURP7741 close to taxon (2.4) with SNP 5988-6868;  
184 Furthermore, taxon (2.4) including *M. liflandii*128FXT and *M. marinum* strain  
185 BB170200 with 2,528 SNPs between them; taxon (2.5) including *M. pseudoshottsii*

186 JCM 15466, *M. pseudoshottsii* and *M. marinum* strain DL240490 with 23-217 SNPs  
187 and this value was up to 1,159 SNPs with *Mycobacterium* sp. 01293.

188

189 **Plasmidome.** The no ribosomal synthesis of mycolactones is encoded by a 174-210-  
190 kb giant plasmid [17, 18]. The mycolactone molecule is composed of an invariant  
191 core-lactone and a side chain which is esterified to a highly unsaturated acyl side  
192 chain, the latter structure varying according to the different MPM strains. Accordingly,  
193 recent studies showed different types of mycolactones A/B, C, D, E, F and G  
194 produced by mycobacteria phenotypically distinct from *M. ulcerans* and not  
195 necessarily associated with Buruli ulcer [1, 25] such as *M. liflandii*, *M. pseudoshottsii*  
196 and some *M. marinum* isolates [26-28]. The pan-genomic analysis carried out on the  
197 44 genomes used in this study showed a clustering of 20 MPMs genomes, these 20  
198 genomes hosting at least one of the plasmids responsible for the synthesis of  
199 mycolactone (pMUM01, pMUM02, pMUM03, pSHT-P). The total plasmid sequence is  
200 represented by three very large genes involved in mycolactone biosynthesis:  
201 (mIsA1: 51 kb, mIsA2: 7 kb and mIsB: 42 kb). MIsA1 and MIsA2 form a complex  
202 synthesising the core-lactone of mycolactone, while MIsB is required for side chain  
203 synthesis [17, 26, 29]. Furthermore, we noted that a taxon (2.1) formed by three  
204 Japanese isolates, one Ugandan isolate and one USA isolate, all harboured the  
205 plasmid pSHT-P. The 166-kb pSHT-P plasmid contains 72 CDSs and was found for  
206 the first time in *M. ulcerans* subsp. *shinshuense* isolated in Japan [29]. The taxon  
207 (2.2), formed by five strains from different African countries, one strain from Australia,  
208 and one strain from the USA, all harbour the plasmid pMUM01. The 174,155-bp  
209 pMUM01 plasmid was revealed in *M. ulcerans* in 2004 by Stinear *et al.*, and encodes  
210 for 81 predicted CDS, with 62.7% GC content [17, 26]. Taxon (2.3), formed by the

211 French Guiana *M. ulcerans* strain CSURP7741 and taxon (2.4) including *M.*  
212 *liflandii*128FXT and *M. marinum* BB170200 isolated from frogs and fish respectively,  
213 all these isolates host the 190-kb plasmid pMUM02 encoding for 95 CDSs with a  
214 62.9% GC, initially described in *M. liflandii* [30]. Taxon (2.5), including *Mycobacterium*  
215 sp. 012931, *M. pseudoshottsii* JCM 15466, *M. pseudoshottsii* and *M. marinum* strain  
216 DL240490, is characterised by plasmid pMUM03 [31]. In addition, we noted the  
217 presence of one or two types of mycolactone synthesis plasmids in addition to the  
218 common plasmid that characterises the taxon. For example, in taxon (2.2), *M.*  
219 *ulcerans* CSURQ0185, *M. ulcerans* CSURP9949 and *M. ulcerans* CSURP9950  
220 contain the plasmid pSHT-P, in addition to plasmid pMUM01 which is characteristic  
221 of this taxon (2.2).

222

223 **Genomospecies.** Combining the ANI, pan-genome and core genome results  
224 enabled us to reclassify the *M. marinum* strain BB170200 and *M. marinum* strain  
225 DL240490 (both isolates from unspecified fish in Israel) as *M. liflandii* included in  
226 taxon (2.4) and *M. pseudoshottii* included in taxon (2.5), respectively (Fig. 2 and Fig.  
227 3). The *Mycobacterium* sp. 012931 had already been published as *Mycobacterium*  
228 sp. related to the *M. marinum* group [32] but in this study we showed that this  
229 mycobacterium is related to the *M. pseudoshottii* taxon (2.5).

230

## 231 **ECOLOGY AND PHENOTYPIC CHARACTERISTICS.**

232 **Geography and Hosts.** The pan-genomic analysis carried out on the 44 genomes  
233 used in this study separated 24 *M. marinum* genomes, forming group (1) and 20  
234 MPMs genomes forming group (2). We reviewed the geographical and ecological  
235 origins and type of mycolactone for each of the 44 isolates here studied, using the

236 NCBI database and the PATRIC web site. Thus, of the 24 genomes of *M. marinum*  
237 isolates, 16 isolates regrouped on the taxon (1.1) were isolated from the USA, the  
238 United Kingdom, Israel, Sweden, and an unspecified European country (10, 3, 1, 1)  
239 genomes, respectively. The unique *M. marinum* strain Davis1 forming taxon (1.2)  
240 was isolated from a human sample from the USA. The taxon (1.3) groups two strains,  
241 *M. marinum* MB2 isolated from fish from Thailand, and *M. marinum* B isolated from a  
242 human sample in France. The two USA isolates, *M. marinum* M and *M. marinum* E11  
243 forming taxon (1.4) were isolated from human and fish samples, respectively. Wille  
244 *M. marinum* MSS2 taxon (1.5), and *M. marinum* DE4576 taxon (1.7) both were  
245 isolated from fish in the USA. Taxon (1.6), formed by *M. marinum* HL1506, was  
246 isolated from fish from China. Thus, 22 *M. marinum* were isolated from fish, of which  
247 12 were marine fish, eight were freshwater fish and two were ubiquitous fish. Three  
248 strains were isolated from humans, including one isolated from a man working in an  
249 aquaculture pool during an outbreak of *M. marinum*, and one *M. marinum* strain 1218  
250 RDel variant of *M. marinum* strain 1218R from in a laboratory in the United Kingdom.  
251 All these strains lack plasmids encoding the synthesis of mycolactone, except for  
252 three strains (*M. marinum* M, *M. marinum* strain Davis1 and *M. marinum* strain  
253 VIMS9) which harbour pMUM03, pMUM01/pSHT-P and pMUM02 respectively.  
254 Intriguingly, *M. marinum* is among the non-tuberculous mycobacterial species most  
255 associated with mycobacterial fish diseases which infect more than 200 species of  
256 freshwater and marine fish [33]. In humans it is responsible for a skin infection after  
257 exposure to freshwater and marine fish over a wide geographical area from the  
258 subarctic to the tropics [34]. *M. marinum* infections are reported in striped bass in the  
259 USA [35], in sturgeon fish from China [36], and in captive-bred Australian lungfish  
260 [37]. In Africa, *M. marinum* has been isolated from the skin of healthy humans and

261 also from the soil [38, 39] and from koi fish exhibiting skin ulcerations on the head in  
262 South Africa [40]. It has also been reported to cause sporadic infections in rainbow  
263 and brown trout in Italy [41]. Water and food that is contaminated by mycobacteria  
264 are the potential sources of infection for fish [42, 43]. Grady AW *et al.* suggest  
265 digestive transmission through the ingestion of contaminated food and cannibalism of  
266 infected fish [44]. Furthermore, in humans, *M. marinum* penetrates the skin with  
267 minor traumas in fishponds and swimming pools [45] as well as skin lesions that  
268 have occurred during the preparation of seafood [46].

269         Within 20 MPMs genomes forming group (2), divided into five taxa, taxon (2.1)  
270 comprises three strains from Japan, one strain from Uganda, one from the USA, and  
271 one from Ghanaian. Taxon (2.2) divided into three taxa includes taxon (2.2.1) that  
272 combines two Australian strain, and USA strain. Three African strains forming taxon  
273 (2.2.2) were isolated from Benin, Ghana and RDC (n=1/ country). One single strain of  
274 *M. ulcerans* CSUR0185, isolated in Côte d'Ivoire forms taxon (2.2.3). The (2.3)  
275 formed by the unique French Guiana strain *M. ulcerans* P7741. Taxon (2.4) includes  
276 two *M. pseudoshottsii* strains from the USA, the *M. marinum* strain DL240490 from  
277 Israel, and the *Mycobacterium* sp. 012931 from China. Some 36 countries with  
278 tropical, subtropical and temperate climates have reported Buruli ulcer, including the  
279 Western Pacific regions of Australia, Malaysia, Papua New Guinea and Sri Lanka ,  
280 the American countries (French Guiana, Mexico and Peru) and African countries,  
281 where the highest concentrations of cases are found in Benin, Cameroon, Côte  
282 d'Ivoire, the RDC and Ghana [13]. To date, the exact source and mode of  
283 transmission of Buruli ulcer remains unknown, although communities associated with  
284 rivers, swamps, wetlands and human-induced changes in the aquatic environment,  
285 especially those created as a result of environmental disturbances such as

286 deforestation, dam construction and agriculture are recognised as sources of  
287 contamination [47]. Like most environmental mycobacteria, *M. ulcerans* proliferates  
288 in aquatic environments with pH values ranging from 5.5 to 6.5 [48]. In Australia, the  
289 PCR investigation of environmental sources of infection revealed the presence of *M.*  
290 *ulcerans* DNA in marsh water used for golf course irrigation [49]. Furthermore, a non-  
291 random collocation of human Buruli ulcer cases was found during the investigation of  
292 faeces samples of ringtail possum and brushtail possum in Australia [50]. In addition,  
293 cases of Buruli ulcer have been described in domestic mammalian species in  
294 Victoria, including long-footed possum koalas, horses, alpaca dogs and cats. All  
295 animal cases were identified in locations where human cases of Buruli ulcer have  
296 been reported [15, 51-53]. In addition, Johnson PDR and collaborators have  
297 suggested the role of mosquitoes in the transmission of *M. ulcerans*, following its  
298 detection in five different species of mosquitoes captured at Point Lonsdale in  
299 Australia [54]. In contrast, in Africa, it was reported that 10.3% of biofilm/plant  
300 samples (3/29), 1.7% of water samples (1/65) and 1.5% of soil samples (2/119) were  
301 positive for the detection of *M. ulcerans* DNA in the Zio and Yoto districts of the  
302 southern maritime region of Togo [55] and 34% aquatic sites in Ghana that were  
303 tested for *M. ulcerans* were reported positive [56]. Furthermore, Aboagye Sy reports  
304 a seasonality around detection of *M. ulcerans* in the environment, reporting 11% and  
305 3% positive environmental samples during the rainy and dry seasons, respectively. In  
306 the same study, the authors reported that *M. ulcerans* DNA was mainly detected in  
307 snails (5/6, 83 %), moss (8/40, 20 %), soil (55/586, 9 %) and vegetation (55/675, 8  
308 %) [57]. *M. ulcerans* was detected in samples of stagnant water, soil, water filtrate  
309 residues, plants and in the aquatic *Heteroptera* from Côte d'Ivoire [58, 59]. An  
310 experimental study demonstrating that water bug bites can transmit *M. ulcerans*

311 infection to mice, because *M. ulcerans* was detected in the salivary glands of water  
312 bugs. Marsollier and collaborators considered water bugs to be potential transient  
313 hosts for *M. ulcerans* [60].

314

315 **Phenotype.** The mycolactone toxin has been strictly associated with *M. ulcerans*,  
316 however, recent studies have shown different types of mycolactones A/B, C, D, E, F  
317 and G produced by other mycobacteria that are phenotypically distinct from *M.*  
318 *ulcerans* and not necessarily associated with Buruli ulcer [1, 25]. These  
319 mycobacteria, isolated from different hosts from different geographical regions, form  
320 the mycolactone-producing mycobacterial complex (MPM), comprising *M. ulcerans*,  
321 *M. liflandii*, *M. pseudoshottsii* and some isolates of *M. marinum* [27, 28]. Thus, the  
322 mycolactone production capacity is broader than previously assumed, and the  
323 genetic diversity of the MPM complex is also greater than previously assumed.  
324 Clustering the geographical origin of isolation, the host of isolation and the plasmid  
325 yielded a representation in agreement with those mentioned above and further  
326 clarified that the *M. marinum* strain BB170200 (PEDI01000001.1) and *M. marinum*  
327 strain DL240490 (PEDJ01000001.1) are two new unnamed species, interestingly  
328 isolated from freshwater and saltwater fish (Figure S3).

329

## 330 **CONCLUSIONS**

331 This study highlighted the profound bias in genome datasets: all the currently named  
332 *M. ulcerans* isolates were made from clinical sources, evidently obscuring the  
333 probable genomic diversity of environmental *M. ulcerans* and underlying the absolute  
334 necessity to boost the culture-based evaluation of environments, while none of the 24  
335 *M. marinum* isolates here investigated were made from the environment. Despite this

336 limitation, our study was able to firmly delineate a group of isolates as *M. ulcerans*  
337 and a group of isolates as *M. marinum*. In between, nine isolates were here  
338 tentatively named *M. marinum*, *M. ulcerans* and *M. guyanensis* sp. nov.; a temporary  
339 classification aimed to stimulate contributing researchers in the field. We report clear-  
340 cut characteristics for the laboratory identification of these two species.

341

## 342 **METHODS**

343 **Whole genome sequence retrieval and analysis.** In order to retrieve the MPM  
344 genome sequences, we consulted the Assembly  
345 (<https://www.ncbi.nlm.nih.gov/assembly/>) and EBI (<https://www.ebi.ac.uk/genomes/>)  
346 databases using the keyword “*Mycobacterium*” in April 2020. Seven genomes of *M.*  
347 *ulcerans* and one genome of *M. marinum* strains from different geographical origin  
348 were then sequenced in our laboratory using MiSeq platform (Illumina, Inc., San  
349 Diego, CA, USA). All these genomes were assembled using SPAdes software  
350 version 3.5.0 [61] and annotated using Prokka version 1.12 [62]. The selected  
351 genomes were then analysed using different comparative genomics tools such as  
352 ANI, DHH, Roary and SNP-distance.

353

354 **Identity cards.** NCBI/PubMed, Google Scholar were used to perform a literature  
355 review as well as the Web of Science website, using the following keywords “Buruli  
356 ulcer”, “*Mycobacterium ulcerans*” “*M. ulcerans* genotype” “genetic diversity of *M.*  
357 *ulcerans*” and “epidemiology of *M. ulcerans*”. The geographic origin and host of  
358 isolation of the strains of these genomes was verified on the Patric and NCBI  
359 websites.

360

361

362 **Figure Legends**

363 **Figure 1.** Average nucleotide identity (ANI) analysis using MORPHEUS (Versatile  
364 matrix visualisation and analysis software), displays the coloured names of isolates  
365 indicating the different taxa of *M. marinum* and MPMs.

366 **Figure 2.** Analysis of the core-genome tree confirming the results of ANI. The 44  
367 genomes of the isolates used in this study are grouped in different taxa represented  
368 by different colours.

369 **Figure 3.** Single-nucleotide polymorphism tree of the 44 isolates studied confirmed  
370 the ANI and the core-genome tree separating the MPMs from *M. marinum* isolates.  
371 The different colours represent the different taxa found during the ANI and core-  
372 genome analysis.

373

374 **Supplementary Data**

375 **Figure S1.** Presentation of GC% of 44 genomes used in this study. 42 genomes  
376 have a GC% of 65.2-65.7%, and the genomes of *M. ulcerans* subsp. *shinshuensis*  
377 strain ATCC 33728 (AP017635.1), and *M. ulcerans* CSURP9951 have 62.3% and  
378 63.4% respectively. The total of these 44 genomes is 260 million bases.

379

380 **Figure S2.** DDH analysis of 44 isolates yielded inconsistent results, despite the use  
381 of the three HDD formulations for the isolates studied.

382

383 **Figure S3.** Identity card representing the geographical origin, host of isolation and  
384 plasmid type of 44 MPM and *M. marinum* genomes investigated in this study.

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389 **References.**

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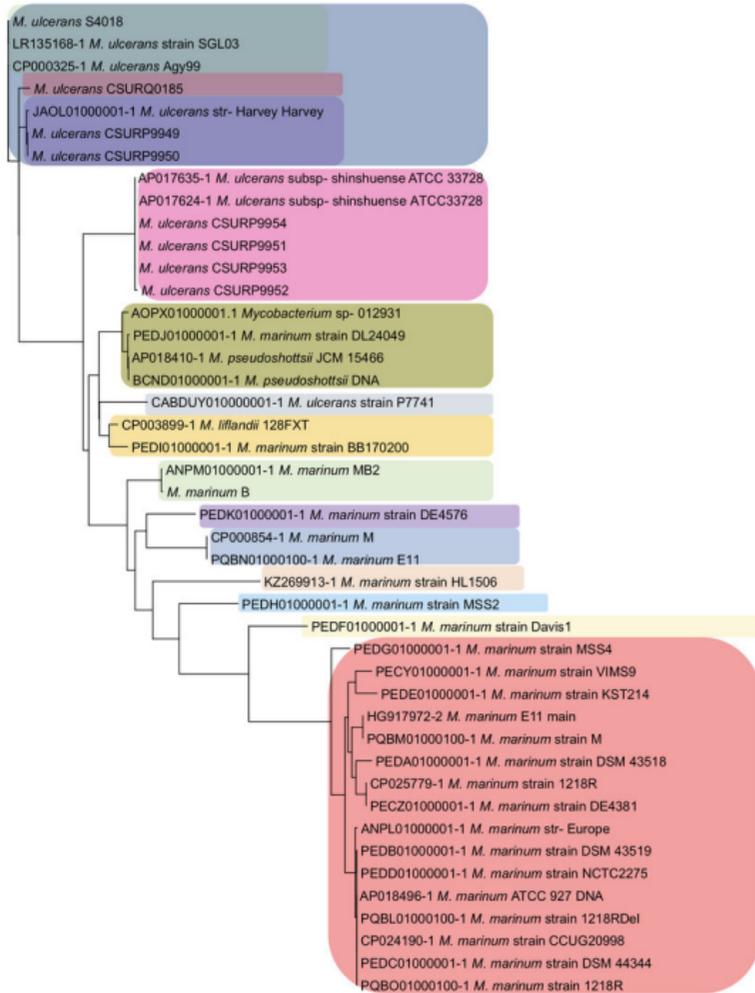
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