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1 **Title:** Genomic polymorphism of *Mycoplasma flocculare* revealed by a newly developed multilocus  
2 sequence typing scheme

3

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15

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17

18

## 19 **Abstract**

20 *Mycoplasma flocculare* is genetically closely related to *M. hyopneumoniae*, the etiologic agent of  
21 porcine enzootic pneumonia, and is frequently isolated with this second species. In this article, we  
22 report on the development of the first multilocus sequence typing (MLST) scheme for *M.*  
23 *flocculare*, based on three genes (*adk*, *rpoB* and *tpiA*). In total, 5,022 bp of sequence were analyzed.  
24 MLST was used to characterize seven *M. flocculare* isolates and the reference strain. Eight distinct  
25 sequence types were defined, showing the great intraspecies variability of *M. flocculare*, and the  
26 high discriminatory power of the new typing method. The relative contribution of recombinations to  
27 the genomic evolution of *M. flocculare* was revealed by calculating the index of association ( $I_A$ :  
28 0.0185). This MLST scheme is now available for the acquisition of new knowledge on *M.*  
29 *flocculare* epidemiology via an online database comprising the DNA sequences of each allele,  
30 available at <http://pubmlst.org/mflocculare/>.

31

## 32 **1. Introduction**

33 *Mycoplasma (M.) flocculare* is commonly described as a commensal agent of the porcine  
34 respiratory tract (Thacker and Minion, 2012). However, this mycoplasmal species can adhere to  
35 respiratory epithelium and cohabits in severe gross pneumonia-like lesions with *M. hyopneumoniae*,  
36 the etiological agent of enzootic pneumonia and the primary agent of the porcine respiratory disease  
37 complex (PRDC). The PRDC is a multifactorial disorder causing financial losses to the pig industry  
38 worldwide (Kobisch and Friis, 1996, Fourour et al., 2018). *M. hyorhinis*, also detected in cases of  
39 pneumonia, can induce polyserositis (pericarditis, pleuritis, peritonitis), arthritis, ear infections,  
40 conjunctivitis and sepsis and may be present in subclinical infections (Thacker and Minion, 2012).  
41 A recent study showed that *M. flocculare* and *M. hyorhinis* were found in extensive gross  
42 pneumonia-like lesions in association with *M. hyopneumoniae*, but their role is not well known  
43 (Fourour et al., 2018). *M. flocculare* and *M. hyopneumoniae* are closely related genetically, and

44 share several virulence factors, raising the question of the potential role of *M. flocculare* as a PRDC  
45 agent (Paes et al., 2018). A comparative analysis between soluble secreted protein repertoires  
46 (secretome) of *M. hyopneumoniae* and *M. flocculare* strains allowed to identify 12 putative  
47 virulence factors in *M. hyopneumoniae* secretome and four putative virulence factors in *M.*  
48 *flocculare* secretome, with two virulence factors in common (Paes et al., 2017). Very recently, this  
49 mycoplasmal association appeared to induce an additive effect and to increase the inflammatory  
50 status of pigs, possibly involving impairment of the immune system (Fourour et al., 2019).

51 *M. flocculare* can be isolated from tracheal and bronchiolar mucus, but isolation and identification  
52 are tedious and time consuming. The difficulty in culturing has led to the development of other  
53 diagnostic assays, including PCR tests (Stakenborg et al. 2006, Fourour et al., 2018). No typing  
54 method has been developed to date for *M. flocculare* strains. Only the comparison of 16S rRNA  
55 genes was performed from four strains and showed a certain genomic diversity (Yamaguti et al.,  
56 2015). Multilocus sequence typing (MLST) scheme is an unambiguous method currently regarded  
57 as the gold standard for typing, able to replace even pulsed-field gel electrophoresis (PFGE)  
58 analysis (Gevers et al., 2005). MLST schemes already published have been used successfully to  
59 characterize *M. hyopneumoniae* and *M. hyorhinis* and demonstrated a great intraspecies diversity  
60 (Mayor et al., 2008; Tocqueville et al., 2014; Kuhnert and Overesch 2014; Trüeb et al., 2016;  
61 Michiels et al., 2017; Feld et al., 2018). This method is based on the nucleotide sequences of  
62 housekeeping genes, in which mutations are assumed to be largely neutral (Selander et al., 1986).  
63 Isolates that share the same sequence type (ST) are assumed to be members of the same clone  
64 (Selander et al., 1986), i.e., they have a recent common ancestor. Data obtained can be used to  
65 address questions about the evolutionary and population biology of bacterial species (Feil et al.,  
66 1999). Moreover, it appears that the MLST scheme can also be used to relate *M. hyorhinis* strains,  
67 according to the pathology observed in pig, to a particular phylogenetic group (in addition to  
68 providing data on genetic diversity), even though the targets are housekeeping genes (Tocqueville et  
69 al., 2014). In this article, we discuss development of the first typing method for *M. flocculare*, an

70 MLST scheme. We also report on findings concerning genomic polymorphism in this mycoplasmal  
71 species, obtained with the new method.

72

## 73 **2. Materials and methods**

74 One reference strain (ATCC 27399) and seven isolates obtained from seven farms in 2016 (**Table**  
75 **1**) were used. All isolates were cultured from lung specimens in Friis liquid medium at  $37 \pm 2$  °C  
76 (Friis, 1975). The isolates derived from a single colony, cloned twice maximum, and the purity was  
77 ensured by multiplex qPCR (Fourour et al., 2018).

78 DNA was extracted from a 12 mL culture with the QIAamp<sup>®</sup> DNA Mini Kit (Qiagen, Courtaboeuf,  
79 France), and quantified with a Qubit<sup>®</sup> 2.0 fluorometer (Invitrogen, Paris, France). DNA was sheared  
80 by sonication using a Bioruptor<sup>®</sup> Plus (Diagenode) apparatus. Libraries were prepared using a  
81 NEBNext<sup>®</sup> Ultra DNA library Prep Kit for Illumina<sup>®</sup> and NEBNext<sup>®</sup> Multiplex Oligos for  
82 Illumina<sup>®</sup>, according to the manufacturer's instructions (New England Biolabs, Evry, France). Size  
83 selection and purification steps were conducted with magnetic beads (Agencourt AMP pure XP  
84 system; Beckman-Coulter, Villepinte, France). Sequencing was performed using MiSeq Illumina  
85 technology (paired-end sequencing 2x150 cycles, MiSeq Reagent kit v2-300 Cycles, Illumina).  
86 Reads were cleaned with Trimmomatic 0.36 (Bolger et al., 2014)  
87 (ILLUMINACLIP:oligos.fasta:2:30:5:1:true LEADING:3 TRAILING:3 MAXINFO:40:0.2  
88 MINLEN:36). Cleaned reads were then aligned versus known references (AFCG01000001.1 to  
89 AFCG01000014.1) with bwa (0.7.15-r1140, Li and Durbin, 2009). Reads were down-sampled to fit  
90 a global coverage estimation of 80×, cleaned with Trimmomatic (same parameters as before), and  
91 submitted to SPAdes (v3.10.0, Nurk et al., 2013). The *de novo* contigs were then submitted to  
92 MEGABLAST (2.2.26, Chen et al., 2015) on a local copy of the NCBI "Nucleotide" databank (nt).  
93 The ordered set of contigs was obtained using MauveReorder (Rissman et al., 2009) on CP007585.1  
94 reference genome. The next step was to annotate the draft genome using the Rapid Annotation  
95 using Subsystem Technology (RAST) server with genetic code 4 (Mold, Protozoan, and

96 Coelenterate Mitochondrial Code and the Mycoplasma/Spiroplasma Code) (Aziz et al., 2008). The  
97 contigs shorter than 200 bp were removed from the analysis. The *Mycoplasma* species of each strain  
98 was verified with the primer sequences described by Fourour *et al.* (2018) in the insilico.ehu.es  
99 website.

100 The new MLST scheme was developed for *M. flocculare* using the complete genes *adk*, *rpoB* and  
101 *tpiA* (**Table 2**). We chose to work on these three genes alone because they have been used  
102 successfully for the development of MLST *M. hyopneumoniae* scheme. These housekeeping genes  
103 were searched with blastn (NCBI BLAST+blastn). For each *M. flocculare* strain (including the  
104 reference strain ATCC 27399, Accession: CP007585.1 and the seven isolates of this study), the  
105 alleles at each of the three loci (e.g. 2,8,7) defined the allelic profile or ST (e.g. ST7) (Calcutt et al.,  
106 2015). The STs were assigned arbitrary numbers in order of description. The allelic profile of *M.*  
107 *flocculare* ATCC 27399 is 1,1,1 (ST1). A web-accessible database has been set up for the MLST  
108 scheme for *M. flocculare* at <http://pubmlst.org/mflocculare/> (Jolley & Maiden 2010). The number of  
109 nucleotide polymorphic sites was determined by using specially designed software, BIGSdb  
110 (<http://pubmlst.org/software/database/bigsgdb/>) (Jolley & Maiden 2010). The degree of clonality  
111 within the data set was estimated by calculating the index of association ( $I_A$ ). The standardized  $I_A$   
112 was used to test the null hypothesis of linkage equilibrium for multilocus data, and therefore  
113 determine the relative contribution of mutation and recombination to the diversity seen by MLST.  
114 An  $I_A$  value significantly different from 0 indicates that a population is clonal (linkage  
115 disequilibrium), while  $I_A$  equal to zero indicates a recombining population structure (linkage  
116 equilibrium). Analysis was performed with the LIAN program ([http://guanine.evolbio.mpg.de/cgi-](http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl)  
117 [bin/lian/lian.cgi.pl](http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl)) by using 10,000 resampling of the data. The calculation of H (representing  
118 genetic diversity) was also performed with LIAN. To investigate whether positive or negative  
119 selection had occurred at the protein level, the average non-synonymous/synonymous substitution  
120 rate ratio (dN/dS) was calculated using START2 software  
121 (<https://pubmlst.org/software/analysis/start2/webstart/start2.jnlp>). Low ratios indicate a lack of or a

122 very limited contribution of environmental selection to the sequence variation in the housekeeping  
123 genes analyzed, which are thus assumed to be suitable for a population genetics study. DnaSP  
124 genetic software, version 5.10.01 was used to calculate Tajima's D statistic in order to test the  
125 neutrality of the observed DNA polymorphisms  
126 (<http://www.ub.es/dnasp/software/dnasp51001.msi>).

127 The MLST sequences concatenated head-to-tail of each strain were aligned and one phylogenetic  
128 tree was drawn on "Phylogeny.fr" (<http://phylogeny.lirmm.fr/phylo.cgi/index.cgi>) with the "One  
129 Click" mode. BURST analysis was carried out to reveal the relationships between MLST sequence  
130 types, and to analyze clonal complexes (<http://pubmlst.org/analysis/>), then to (i) divide strains into  
131 groups according to their allelic profiles, and (ii) count the number of single locus variants (SLVs),  
132 double locus variants (DLVs), and satellites (SATs) for each ST, and identify the potential ancestral  
133 type (AT). The original BURST algorithm was previously developed (Feil et al., 2004). The version  
134 used was adapted as a plugin for BIGSdb database software by Keith Jolley  
135 (<http://pubmlst.org/software/database/bigsgdb/>) (Jolley & Maiden 2010).

136 The relationships between ST patterns, ST phylogenetic groups, and BURST groups detected in this  
137 study and the mycoplasmal combinations or pneumonia-like macroscopic lesion scores observed on  
138 the origin farms (Fourour et al. 2018) were analyzed. The statistical analyses were performed using  
139 the Fisher exact test ( $n \leq 5$ ) on independence in two-by-two tables  
140 (<https://marne.u707.jussieu.fr/biostatgv/?module=tests/fisher>). Differences were considered to be  
141 significant when  $p < 0.1$ .

142

### 143 **3. Results and discussion**

144 The assemblies of *M. flocculare* isolates yielded 29 to 71 contigs, covering a total of 739,183 to  
145 782,983 bp (mean genome size,  $762,578 \pm 13,290$  bp), with an  $N_{50}$  value of 67,566 to 123,263 bp  
146 (mean  $N_{50}$  value,  $82,858 \pm 18,873$  bp), an average coverage of 74.8× to 93.4× (mean coverage,  $83.8$   
147  $\pm 7.7$ ×), and an overall G+C content of 29.0% to 29.6% (mean G+C content,  $29.1 \pm 0.2$ %). The

148 seven *M. flocculare* isolates yielded 620 to 669 coding sequences (CDSs), whereas the previously  
149 published *M. flocculare* ATCC 27399 genome contained only 585 CDSs (Siqueira et al., 2013).  
150 One hypothesis would be that this difference might be related to the presence of more CDSs  
151 encoding, for example, virulence factors in our current isolates. The reference strain cultivated  
152 many times on culture medium may have lost non-essential genetic elements for *in vitro* growth,  
153 while these elements were essential to the colonization of the animal. For our seven *M. flocculare*  
154 isolates and the reference strain ATCC27399, the discriminatory ability of the different loci,  
155 measured as number of alleles, varied from six (*adk*) to eight (*rpoB* and *tpiA*) (**Table 2**). The  
156 genetic diversity (H) obtained from the three genes varied from 0.9286 (*adk*) to 1.000 (*rpoB* and  
157 *tpiA*) (**Table 2**). The standardized  $I_A$  was calculated at 0.0185. Most polymorphisms resulted in  
158 nonsynonymous substitutions, with the ratio of nonsynonymous to synonymous substitutions  
159 (dN/dS) varying from 0.0164 (for *tpiA*) to 0.1482 (for *adk*), and Tajima's D values varying from -  
160 0.351 (for *adk*) to 0.459 (for *tpiA*) (with none of these values significantly deviating from zero,  $p >$   
161 0.10). These values show that the genetic variation was roughly in linkage equilibrium, and seemed  
162 to be due to recombination (with mutation playing a much smaller role) (Haubold et al., 1998).  
163 Homologous recombination was also high for *M. hyopneumoniae* and *M. hyorhinitis* (Mayor et al.,  
164 2008; Tocqueville et al., 2014). In *M. hominis*, the frequency of recombination is not correlated  
165 with the level of variability, and recombination does not induce more variation in the genes but  
166 rather shuffles the existing mutations, thereby creating new alleles (Søgaard et al., 2002).  
167 Recombination in *M. hyopneumoniae*, *M. hyorhinitis* and *M. flocculare* is particularly interesting  
168 since no phages or plasmids have been described in these species. However, for *Mycoplasma*  
169 species sharing the same hosts, a significant number of genes undergoing horizontal transfer have  
170 been described (Sirand-Pugnet et al., 2007).

171 This MLST scheme of three housekeeping genes, *adk*, *rpoB* and *tpiA* (concatenated 5022 nt) was  
172 able to type seven *M. flocculare* isolates and the *M. flocculare* reference strain and to identify eight  
173 distinct sequence types, showing the great intraspecies variability of *M. flocculare*, and offering a



174 very highly discriminatory typing method. The MLST scheme for *M. hyopneumoniae* was also  
175 based on the same three housekeeping genes (Mayor et al., 2008). This simple scheme has been  
176 found to be sufficient for epidemiological investigations of *M. hyopneumoniae*, and also seems to  
177 be sufficient for *M. flocculare*. However, more strains should be typed to confirm these preliminary  
178 results. Our MLST scheme confirms that sequence variations occur within housekeeping genes,  
179 which indicates that the core genome is also variable.

180 The genetic relationships between the eight *M. flocculare* STs are shown in the phylogram in  
181 **Figure 1**. Three ST groups, named “a” to “c”, were identified. The BURST analysis revealed two  
182 groups of two *M. flocculare* isolates, BURST groups A and B. BURST group A was composed of  
183 DLVs ST2 and ST7. BURST group B was composed of DLVs ST3 and ST4. ST1, 5, 6 and 8 were  
184 not linked to groups and were singletons. The BURST analysis did not highlight a clonal complex  
185 for *M. flocculare* strains, whereas several clonal complexes were identified for *M. hyopneumoniae*  
186 and *M. hyorhinis* (Mayor et al. 2008; Tocqueville et al., 2014; Kuhnert and Overesch 2014; Trüeb et  
187 al., 2016; Michiels et al., 2017; Feld et al., 2018). *Helicobacter pylori* and *Staphylococcus aureus*  
188 populations, also with rich histories of interstrain recombinations, are structured in clonal  
189 complexes (Achtman et al., 1999; Day et al., 2001). The analysis of more *M. flocculare* isolates  
190 should make it possible to determine whether such clonal structures are found in this species.

191 This is the first time that *M. flocculare* strains isolated from pneumonia have been typed. All *M.*  
192 *flocculare* isolates from farms with a mean gross pneumonia-like lesions score above 5/28 are in the  
193 same ST group (group “b”) (**Table 3**), showing genomic homogeneity. However, with a *p* value of  
194 0.142, this result must be confirmed. No relationships between the combination of *M. flocculare*  
195 with *M. hyopneumoniae* and/or *M. hyorhinis* and ST groups or BURST groups were observed (*p* >  
196 0.1) (**Table 3**).

197 An online database for the *M. flocculare* MLST scheme, comprising DNA sequences of each allele,  
198 was developed and is available in Pubmlst (<http://pubmlst.org/mflocculare/>). New data concerning  
199 *M. flocculare* genomes, obtained or not by next generation sequencing, can now be included in the

200 database for comparison between laboratories. This genomic approach, certainly coupled with other  
201 omics techniques (proteomic, secretomic), will facilitate epidemiological investigations and the  
202 acquisition of new knowledge about the porcine respiratory disease complex, in connection with the  
203 presence of *M. flocculare*.

204

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210

## 211 **Conflict of interest**

212 The authors have no conflicts of interest.

213

## 214 **Authors’ contributions**

215 The experiments were designed by SF, IK and CMC. The experiments were performed by SF and  
216 VT. The data were analyzed by SF, PL, FT and CMC. Reagents/materials/analysis tools were  
217 contributed by SF, VT, IK and CMC. Contributions to the writing of the manuscript were made by  
218 SF, PL, FT, VT, GBAV, IK and CMC.

219

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328

329 **Table 1.** Main characteristics of the eight *M. flocculare* (MF) strain/isolates and sequence types  
 330 identified by MLST.

Isolate	Mycoplasmal combinations <sup>a</sup>	Mean pneumonia score (/28) <sup>b</sup>	ST profile <sup>c</sup>	ST <sup>d</sup>
ATCC <sup>e</sup> _27399	NK <sup>f</sup>	NK	1,1,1	1
MF11	MHP/MHR/MF	4.9	2,2,2	2
MF12	MF/MHR	2	4,6,3	3
MF18	MHP/MHR/MF	8.9	4,3,4	4
MF22	MF/MHP	2.3	3,7,5	5
MF29	MHP/MHR/MF	7.3	5,4,6	6
MF30	MF/MHR	1.4	2,8,7	7
MF33	MF/MHP	6.3	6,5,8	8

331 <sup>a</sup> Mycoplasmal combination observed in pig lungs at the farm level (Fourour et al., 2018). MHP: *M. hyopneumoniae*, MHR: *M.*  
 332 *hyorhinis*

333 <sup>b</sup> Mean score of gross pneumonia-like lesions by farm, estimated as previously described by Madec and Kobisch (1982)

334 <sup>c</sup> Results of *M. flocculare* typing obtained with the complete genes *adk*, *rpoB*, and *tpiA* using the MLST scheme described in this  
 335 study.

336 <sup>d</sup> ST: sequence type

337 <sup>e</sup> ATCC: American Type Culture Collection, Rockville, USA

338 <sup>f</sup> NK: Not known

339

340

341 **Table 2.** Characteristics of the three genes used in the *M. flocculare* (MF) typing scheme

Locus	Putative function of gene product	Position <sup>a</sup>	Seq. size (bp)	No. of alleles identified	No. of polymorphic nucleotide sites (%)	Genetic diversity (H)	$d_N/d_S$ <sup>b</sup>	Tajima's D <sup>c</sup>
<i>adk</i>	Adenylate kinase	555204	639	6	6 (0.94)	0.9286	0.1482	-0.351
<i>rpoB</i>	RNA polymerase $\beta$ -subunit	675017	3654	8	44 (1.20)	1.0000	0.0306	-0.126
<i>tpiA</i>	Triose-phosphate isomerase	120812	729	8	10 (1.37)	1.0000	0.0164	0.459

342 <sup>a</sup>Position in MF ATCC 27399 (accession CP007585.1)343 <sup>b</sup>Ratio of nonsynonymous to synonymous mutations344 <sup>c</sup>Tajima's D, none of the values significantly deviated from zero ( $p > 0.10$ )

345



346

347 **Table 3.** Distribution of ST patterns, ST groups, and BURST groups in relation to *M. flocculare* isolate  
 348 origins.

	n	Mycoplasmal combinations <sup>a</sup>				Pneumonia-like lesion score <sup>b</sup>		
		MHP/MHR/MF	MHP/MF	MHR/MF	NK <sup>c</sup>	≤5/28	>5/28	NK <sup>c</sup>
No. of strains/isolates	8	3	2	2	1 <sup>d</sup>	4	3	1 <sup>d</sup>
No. of STs	8	3	2	2	1	4	3	1
No. of strains/isolates in ST groups								
	a	1	0	0	1	0	1	0
	b	4	2	1	1	0	1	3
	c	3	1	1	0	1	2	0
No. of strains/isolates in BURST groups								
	group 1	2	1	0	1	0	2	0
	group 2	2	1	0	1	0	1	1
	singletons	4	1	2	0	1	1	2

349 <sup>a</sup> Mycoplasmal combinations observed on the farms of origin (Fourour et al. 2018)

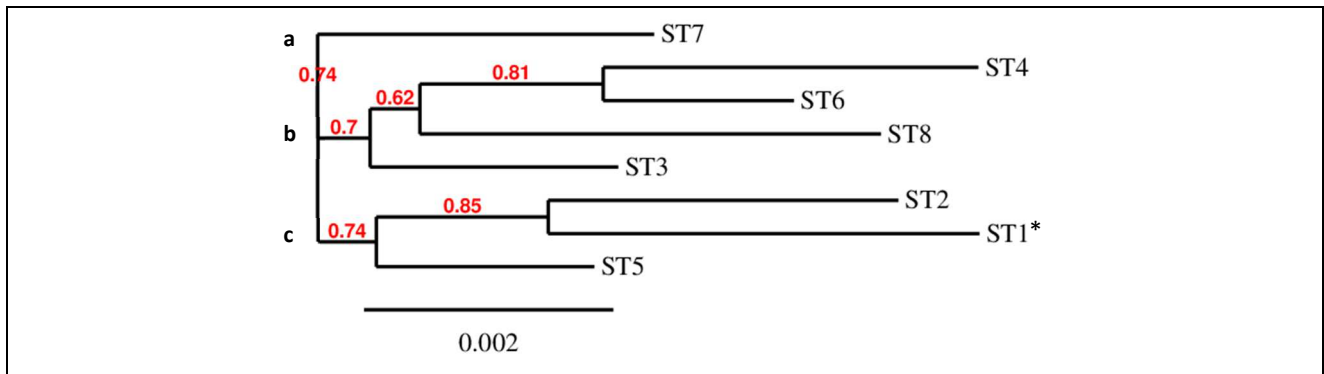
350 <sup>b</sup> Mean score of gross pneumonia-like lesions by farm, estimated as previously described by Madec and Kobisch (1982)

351 <sup>c</sup> NK: not known

352 <sup>d</sup> *M. flocculare* strain ATCC 27399 with genome available in GenBank (accession CP007585.1)

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354



355

356 **Figure 1.** Genetic relationships between seven *M. flocculare* STs and the reference ST1\* (strain *M.*  
357 *flocculare* ATCC 27399), as estimated by clustering analysis of STs revealed by MLST, with loci  
358 of genes *adk*, *rpoB* and *tpiA* concatenated (5,022 bp) for *M. flocculare*. The phylograms were  
359 constructed using tools at [www.phylogeny.fr](http://www.phylogeny.fr) and the “one click” mode. Bootstrap support values  
360 are in red.

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