A comparative genomics and immunoinformatics approach to identify epitope-based peptide vaccine candidates against bovine hemoplasmosis

Rosa Estela Quiroz-Castañeda[1]*, Hugo Aguilar-Díaz[2], Diana Laura Flores-García[3], Fernando Martínez-Ocampo[4], Itzel Amaro-Estrada

[1]Unidad de Anaplasmosis, Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP. Carretera Federal Cuernavaca-Cuautla No. 8534, Col. Progreso, 62550, Jiutepec, Morelos, México.

[2]Unidad de Artropodología, Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP. Carretera Federal Cuernavaca-Cuautla No. 8534, Col. Progreso, 62550, Jiutepec, Morelos, México.

[3] Universidad Politécnica del Estado de Morelos, Paseo Cuauhnahuac 566, Lomas del Texcal, C.P. 62574 Jiutepec, Morelos, México.

[4]Laboratorio de Estudios Ecogenómicos, Centro de Investigación en Biotecnología, Universidad Autónoma del Estado de Morelos, Av. Universidad No. 1001, Col. Chamilpa, 62209, Cuernavaca, Morelos, México.

*Corresponding author

E-mail: requiroz79@yahoo.com.mx

Abstract

Mycoplasma wenyonii and '*Candidatus* Mycoplasma haemobos' have been described as major hemoplasmas that infect cattle worldwide. Currently, three bovine hemoplasma genomes are known. The aim of this work was to know the main genomic characteristics and the evolutionary relationships between hemoplasmas, as well as to provide a list of epitopes identified by immunoinformatics that could be used as vaccine candidates against bovine hemoplasmosis. So far, there is not a vaccine to prevent this disease that impact economically in cattle production around the world.

In this work, we used comparative genomics to analyze the genomes of the hemoplasmas so far reported. As a result, we confirm that '*Ca.* M haemobos' INIFAP01 is a divergent species from *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts. Although both strains of *M. wenyonii* have genomes with similar characteristics (length, G+C content, tRNAs and position of rRNAs) they have different structures (alignment coverage and identity of 51.58 and 79.37%, respectively).

The correct genomic characterization of bovine hemoplasmas, never studied before, will allow to develop better molecular detection methods, to understand the possible pathogenic mechanisms of these bacteria and to identify epitopes sequences that could be used in the vaccine design.

Introduction

Hemotrophic mycoplasmas (hemoplasmas) are a group of erythrocytic pathogens of the *Mollicutes* class that infect a wide range of vertebrate animals [1,2]. At first, these small and uncultivable *in vitro* bacteria were classified as species of the genera *Haemobartonella and Eperythrozoon*, within the *Anaplasmataceae* family and *Ricketsiales* order [1]. However, the

46 genetic analysis of 16S ribosomal RNA (rRNA) gene and morphologic similarities showed that
47 these bacteria are closely related to the *Mycoplasma* genus [2,3]. In 2001, the formal proposal
48 was presented to transfer these organisms to genus *Mycoplasma*, within *Mycoplasmataceae*
49 family [4]. Currently, 12 hemoplasma genomes have been identified in the GenBank database,
50 including *Mycoplasma wenyonii* strains and *Candidatus* Mycoplasma haemobos [5–7]. These
51 hemoplasma genomes have provided relevant information about possible pathogenic
52 mechanisms, metabolism and divergences when compared to other *Mycoplasma* species [2].
53 To date, *M. wenyonii* and *Ca.* M. haemobos have been described as major hemoplasmas that
54 infect cattle worldwide [8–10]. In cattle, acute hemoplasma infections are rare but are
55 characterized by anemia, fever, depression and diarrhea [11,12]. Chronic bovine hemoplasma
56 infections have been associated with variable clinical signs, including low-grade bacteremia,
57 weight loss, decreased milk production, reduced calf birth weight, pyrexia, scrotal and hind limb
58 edema, infertility and reproductive inefficiency, and consequently, the bovine hemoplasmas have
59 caused major economic losses worldwide, mainly when they associate with pathogens of genus
60 *Anaplasma* or *Babesia* [13–15]. In addition, latent and asymptomatic infections have also been
61 reported [14]. Single infections or coinfections between *M. wenyonii* and '*Ca.* M. haemobos' are
62 being reported [16–18]. However, reports of the genomic characterization of bovine
63 hemoplasmas are scarce. So far, two genomes of this species were reported: *M. wenyonii*
64 Massachusetts [5] and *M. wenyonii* INIFAP02 [7] and only one genome of this species was
65 reported: '*Ca.* M. haemobos' INIFAP01 [6]. On the other hand, computational *in silico* tools
66 have been used to design vaccines by rational and cost effective manner, this strategy has several
67 advantages, including prolonged immunity, elimination of unspecific responses and cost-and
68 time-effectiveness [19,20]. In this sense, immunoinformatics is an effective tool that helps to

predict and to identify immunogenic sequences and the epitopes that could be recognized by antibodies that induce immune responses [21].

In this work, we analyzed, for the first time, the genomic characteristics and the evolutionary relationships between bovine hemoplasmas. Also, we performed a immunoinformatic analysis to elucidate B-cell epitopes found in several proteins of *M. wenyonii* and *Ca*. M. haemobos that could be used as potential vaccine candidates to prevent bovine hemoplasmosis.

Materials and methods

Genome sequences and annotation

The 12 hemoplasma genomes that infect different hosts, included in this study and reported in the GenBank database (https://bit.ly/314fOre), are listed in Table S1. In Mexico, the Anaplasmosis Unit (CENID-SAI, INIFAP) has reported the draft genomes of two Mexican strains of hemoplasmas that infect cattle, '*Ca.* Mycoplasma haemobos' INIFAP01 [6] and *M. wenyonii* INIFAP02 [7]. The general features of 12 hemoplasma genomes were obtained using the QUAST (Quality Assessment Tool for Genome Assemblies) (v5.0.2) program [22] with default settings.

All genomes were annotated automatically to predict the coding sequences (CDS) using the RAST (Rapid Annotation using Subsystem Technology) (v2.0) server (https://bit.ly/2XjTTey) [23] with the Classic RAST algorithm.

The mapping of ribosomal genes (rRNA) was done based on the information reported in NCBI database of genomes of *M. wenyonii* Massachusetts, *M. wenyonii* INIFAP02 and *Ca*. M. haemobos INIFAP01 (NC_018149.1; NZ_QKVO00000000.1; and LWUJ00000000.1, respectively). Transfer (tRNA) RNA genes was carried out using ARAGORN (v1.2.38)

(https://bit.ly/3k1R2QT) server [24].The sequence and length of 16S and 23S rRNA genes was obtained from the RNAmmer (v1.2) (https://bit.ly/3glQKCj) server [25].

Phylogenetic and pan-genomic analysis

For the phylogenetic reconstruction, the 16S rRNA gene sequences of 15 bovine hemoplasmas were aligned with 22 downloaded rRNA gene sequences of other hemoplasma species and two downloaded rRNA gene sequences of the genus *Ureaplasma*, which were obtained from the GenBank database (https://bit.ly/314fOre) using the nucleotide BLAST (Blastn) suite (https://bit.ly/3k2Wkvs) [26]. Multiple alignments between 39 16S rRNA gene sequences were made using the MUSCLE (v3.8.31) program [27]. The jModelTest (v2.1.10) program [28] was used to select the best model of nucleotide substitution with the Akaike information criterion. The phylogenetic tree was estimated under the Maximum-Likelihood method using the PhyML (v3.1) program [29] with 1,000 bootstrap replicates. The phylogenetic tree was visualized and edited using the FigTree (v.1.4.4) program (https://bit.ly/39ROMXV).

Two pan-genomic analyzes were performed using the GET_HOMOLOGUES (v3.3.2) software package [30] with the following options: i) among the 12 hemoplasma genomes; and ii) among the three genomes of bovine hemoplasmas. Briefly, the FAA (Fasta Amino Acid) annotation files of hemoplasma genomes were used as input files by the GET_HOMOLOGUES software package. The get_homologues.pl and compare_clusters.pl Perl scripts were used to compute a consensus pan-genome, which resulting from the clustering of the all-against-all protein BLAST (Blastp) results with the COGtriangles and OMCL algorithms. The pan-genomic analysis was performed using the binary (presence-absence) matrix.

Comparative genomics

The average nucleotide identity (ANI) values of 12 hemoplasma genomes were calculated using the calculate_ani.py Python script (https://bit.ly/2X96hho) with the BLAST-based ANI (ANIb) algorithm. Ultimately, the level of conserved genomic sequences of bovine hemoplasmas was visualized by alignment the genomes of Mexican strains ('*Ca.* M. haemobos' INIFAP01 and *M. wenyonii* INIFAP02) against the reference genome of *M. wenyonii* Massachusetts, using the NUCmer program of MUMmer (v3.0) software package (Kurtz et al., 2004) to get the positions of nucleotides that were aligned; and Circos (v0.69-9) software package [32]. The circular comparative genomic map of bovine hemoplasmas was edited with Adobe Photoshop CC (v14.0 x64) program.

Prediction of antigenic proteins

After RAST annotation, we identified several proteins of the subsystems, including virulence, disease and defense, cell division and cell cycle, fatty acids, lipids and isoprenoids, regulation and cellular signaling, stress response, and DNA metabolism. To predict antigenicity, the sequence of 11 proteins of *M. wenyonii* and 12 proteins of *Ca*. M. haemobos were submitted to VaxiJen v2.0 server (http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with default parameters.

Prediction of subcellular localization and stability of the proteins

Predicted antigenic proteins of *M. wenyonii* and *Ca.* M. haemobos were submitted to the prediction of secondary structure server Raptor X (http://raptorx.uchicago.edu/).

Linear B-cell epitope prediction and three-dimensional modelling

B-Cell epitopes were predicted using BCEpred (http://crdd.osdd.net/raghava/bcepred/), and Predicting Antigenic Peptides tool (http://imed.med.ucm.es/Tools/antigenic.pl).

The PHYRE2 server was used to predict the tridimensional structure of the proteins of both hemoplasmas. Phyre2 PDB files were visualized with Protter tool.

Results

General features of genomes

The major genomic features of hemoplasmas are shown in Table 1.

Table 1. General features of 12 hemoplasma genomes.

Organism	Assembly level	Length (bp)*	G+C content (%)*	CDS **	rRNAs#	tRNAs##
'*Ca.* M. haemobos' INIFAP01	18 contigs	935,638	30.46	1,180	3	31
***M. haemocanis* Illinois**	Chromosome	919,992	35.33	1,234	3	31
***M. haemofelis* Langford 1**	Chromosome	1,147,259	38.85	1,595	3	31
***M. haemofelis* Ohio2**	Chromosome	1,155,937	38.81	1,650	3	31
'*Ca.* M. haemolamae' Purdue	Chromosome	756,845	39.27	1,045	3	33
'*Ca.* M. haemominutum' Birmingham 1	Chromosome	513,880	35.52	587	3	32
***M. ovis* Michigan**	Chromosome	702,511	31.69	918	4	32
***M. parvum* Indiana**	Chromosome	564,395	26.98	578	3	32
***M. suis* Illinois**	Chromosome	742,431	31.08	914	3	32
***M. suis* KI3806**	Chromosome	709,270	31.08	856	3	32
***M. wenyonii* INIFAP02**	37 contigs	596,665	33.43	678	3	32
***M. wenyonii* Massachusetts**	Chromosome	650,228	33.92	727	3	32

CDS: coding sequences; *data obtained with the QUAST program; **data obtained with the RAST server; #data obtained with the RNAmmer server; and ##data obtained with the ARAGORN server.

Of the 12 hemoplasma genomes, ten genomes are assembled in a single chromosome and two draft genomes are assembled in contigs. The genomic features of hemoplasmas allow them to be separated into two groups: the group 1 (previously named *Haemobartonella*) consists of four genomes of '*Ca.* M. haemobos', *M. haemocanis* and *M. haemofelis* species, that have a length

149 from 0.9 to 1.1 Mb, a number of CDS from 1,180 to 1,650, and specifically 31 tRNA genes; and
150 the group 2 (previously named *Eperythrozoon*) consists of eight genomes of '*Ca.* M.
151 haemolamae', '*Ca.* M. haemominutum', *M. ovis*, *M. parvum*, *M. suis* and *M. wenyonii* species,
152 that have a length from 0.5 to 0.7 Mb, a number of CDS from 578 to 1,045; and 32 or 33 tRNA
153 genes.
154 The mapping of rRNA genes shows that hemoplasmas are also separated into two groups. The
155 four genomes of group 1 contain one copy of 16S-23S-5S rRNA operon (Fig 1A). The 16S
156 rRNA gene sequences of group 1 have a length from 1,459 to 1,460 bp. Conversely, seven
157 genomes of group 2 contain one copy of 16S rRNA gene with a length from 1,479 to 1,499 pb,
158 which is separated from one copy of 23S-5S rRNA operon (Fig 1B and 1C). Also, the genome of
159 *M. ovis* Michigan of group 2 contains two copies of 16S rRNA gene with lengths of 1,467 and
160 3,219 bp, which are separated from each other, and they are separated from the one copy of 23S-
161 5S rRNA operon (Fig 1D).
162 **Fig 1. Mapping of rRNA genes of hemoplasmas.** A) Genomes of '*Ca.* M. haemobos'
163 INIFAP01, *M. haemocanis* Illinois, *M. haemofelis* Langford 1 and *M. haemofelis* Ohio2 of group
164 1 contain one copy of 16S-23S-5S rRNA operon. B) Genomes of **'***Ca.* M. haemolamae**'** Purdue,
165 **'***Ca.* M. haemominutum**'** Birmingham 1, *M. suis* Illinois, *M. suis* KI3806, *M. wenyonii*
166 INIFAP02 and *M. wenyonii* Massachusetts of group 2 contain one copy of 16S rRNA gene
167 which is separate from one copy of 23S-5S rRNA operon in different chain. C) Genome of *M.*
168 *parvum* Indiana of group 2 contains one copy of 16S rRNA gene which is separate from one
169 copy of 23S-5S rRNA operon in the same chain. D) Genome of *M. ovis* Michigan of group 2
170 contains two copies of 16S rRNA gene which are separated from each other, and they are

171 separated from the one copy of 23S-5S rRNA operon in the same chain. The 16S, 23S and 5S
172 rRNA genes are represented by blue, green and red arrows, respectively.
173
174 The 16S rRNA gene sequence of '*Ca.* M. haemobos' INIFAP01 has alignment coverage of 82-
175 98% and identity of 98.71-99.93% with '*Ca.* M. haemobos', '*Ca.* M. haemobos' clone 307, '*Ca.*
176 M. haemobos' clone 311 and '*Ca.* M. haemobos' isolate cattle no. 18. Additionally, 16S rRNA
177 gene sequence of '*Ca.* M. haemobos' INIFAP01 has alignment coverage of 99% and identity of
178 81.83 and 81.73% with *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts, respectively.
179 On the other hand, the genomes of *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts are
180 very similar in length and G+C content to each other, and they have the same number of tRNA
181 genes and distribution of rRNA genes. However, the 16S rRNA gene sequence of *M. wenyonii*
182 INIFAP02 has alignment coverage of 100% and identity of 97.57% with *M. wenyonii*
183 Massachusetts. Additionally, 16S rRNA gene sequence of *M. wenyonii* INIFAP02 has: i)
184 alignment coverage of 91-98% and identity of 99.24-99.93% with *M. wenyonii* isolate Fengdu,
185 *M. wenyonii* clone 1, *M. wenyonii* isolate ada1 and *M. wenyonii* isolate C124; and ii) alignment
186 coverage of 90-98% and identity of 97.50-97.87% with *M. wenyonii* strain CGXD, *M. wenyonii*
187 isolate B003, *M. wenyonii* isolate C031 and *M. wenyonii* strain Langford.
188 **Phylogenetic and pan-genomic analyzes**
189 The model of nucleotide substitution of the phylogenetic tree that is based on 16S rRNA gene of
190 hemoplasmas was GTR+I+G. The phylogenetic tree shows that groups 1 and 2 of hemoplasma
191 species are separated into different clades (Fig 2). The clade 1 (blue lines) contains two sub-
192 clades: i) '*Ca.* M. haemobos' species; and ii) *M. haemocanis* and *M. haemofelis* species. The
193 clade 2 (red lines) also contains two subclades: i) '*Ca.* M. haemolamae', '*Ca.* M.

haemominutum', *M. ovis* and *M. wenyonii* species; and ii) *M. parvum* and *M. suis* species. The phylogenetic tree topology shows a divergence between two groups of hemoplasmas.

Fig 2. Phylogenetic relationships based on the 16S rRNA genes of group 1 (blue lines) and 2 (red lines) of hemoplasma species. The phylogenetic tree was obtained using the PhyML program with the Maximum-Likelihood method and 1,000 bootstrap replicates. Bootstrap values (>50%) are displayed in the nodes. The model of nucleotide substitution was GTR+I+G. The INIFAP01 and INIFAP02 Mexican strains of bovine hemoplasmas are shown in blue and red letters, respectively. GenBank accession numbers are shown in square brackets.

Pan-genomic analysis among the 12 hemoplasmas shows that the core, soft core, shell and cloud genomes are composed of 110, 146, 787 and 3,099 gene clusters, respectively (Figs S1 and S2). Additionally, the core genomes of groups 1 and 2 of hemoplasmas are composed of 236 and 149 gene clusters, respectively.

Pan-genomic analysis among the three genomes of bovine hemoplasmas shows that the core genome is composed of 154 gene clusters. Also, the two genomes of *M. wenyonii* species share 273 gene clusters. Moreover, '*Ca*. M. haemobos' INIFAP01, *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts contain 312, 190 and 157 unique gene clusters, respectively.

Comparative genomics

ANIb values between different hemoplasma species show that alignment coverage is less than 79% (Fig 3A and Table S2); and identity is less than 83% (Fig 3B and Table S3).

Fig 3. Heatmaps of BLAST-based average nucleotide identity (ANIb) values of 12 hemoplasma genomes. A) Heatmap of ANIb values of alignment coverage. B) Heatmap of

ANIb values of identity. Color intensity increases from white to deep blue when ANIb values approach from 0.0 to 1.0 (0 - 100%), respectively.

Also, ANIb values show that '*Ca.* M. haemobos' INIFAP01 has an alignment coverage of 0.46 and 0.31%; and identity of 74.12 and 74.16% with *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts, respectively. Moreover, ANI values between the same species show that: i) *M. haemofelis* genomes have an alignment coverage and identity of 97.65 and 97.41%, respectively; ii) *M. suis* genomes have an alignment coverage and identity of 95.13 and 97.63%, respectively; and iii) *M. wenyonii* genomes have an alignment coverage and identity of 51.58 and 79.37%, respectively.

The circular map (Fig 4) shows that '*Ca.* M. haemobos' INIFAP01 genome only has three small regions (red lines highlighted with green marker in inner track) greater than 78% identity that were aligned with *M. wenyonii* Massachusetts genome (black circle in outer track). Also, the circular map shows that *M. wenyonii* INIFAP02 has few regions (blue lines in intermediate track) greater than 78% identity that were aligned with *M. wenyonii* Massachusetts genome.

Fig 4. Circular map of *M. wenyonii* Massachusetts and'*Ca.* M. haemobos' INIFAP01 genomes.

Selection and prediction of B-cell epitopes in proteins

The sequences of 11 proteins of *M. wenyonii* and 12 proteins of *Ca*. M. haemobos were submitted to VaxiJen. The prediction of Antigen/Non Antigen for each selected protein is shown in Table 2.

Table 2. Prediction of antigenicity of proteins of *Ca.* M. haemobos and *M. wenyonii.*

Candidatus Mycoplasma haemobos INIFAP01

Classification	Prediction score as antigen (Vaxijen)
RAST Category: Virulence, disease, and defense	
DNA gyrase subunit B	0.5352 (Antigen)
DNA gyrase subunit A	0.4373 (Antigen)
SSU ribosomal protein S7p	0.518 (Antigen)
Translation Elongation factor G	0.5399 (Antigen)
Translation Elongation factor Thermo unstable (Tu)	0.4268 (Antigen)
SSU ribosomal protein S12p	0.7537 (Antigen)
DNA-directed RNA polymerase beta subunit	0.3781 (Antigen)
DNA-directed RNA polymerase	0.4488 (Antigen)
RAST Category: Division and Cell Cycle	
ProteinTsaD/Kae1/Qri7	0.3846 (Non Antigen)
RNA polymerase sigma factor RpoD	0.3857 (Non Antigen)
DNA primase	0.3009 (Non Antigen)
RAST Category: Fatty acids, Lipids and Isoprenoids	
Cardiolipin synthase	0.3463 (Non Antigen)
RAST Category: Stress Response	
Manganase superoxide dismutase	0.3487 (Non Antigen)
Mycoplasma wenyonii INIFAP02	
RAST Category: Virulence, disease, and defense	
Ribosomal protein SSU S7p	0.5435 (Antigen)
Translation Elongation factor G	0.5650 (Antigen)
Translation Elongation factor Thermo unstable (Tu)	0.4359 (Antigen)
Ribosomal protein SSU S12p	0.7774 (Antigen)
Ribosomal protein LSU L35p	0.5491 (Antigen)

Translation Initiation factor 3	0.4488 (Antigen)
Ribosomal protein LSU L20p	0.3668 (Antigen)

RAST Category: Division and Cell Cycle

Protein TsaD/Kae1/Qri7	0.3954 (Non Antigen)
RNA polymerase sigma factor RpoD	0.3979 (Non Antigen)
DNA primase	0.3929 (Non Antigen)

RAST Category: Fatty acids, lipids and isoprenoids

Cardiolipin synthase	0.3305 (Non Antigen)

The collection of B-cell epitopes (linear antigens) predicted with Predicting Antigenic Peptides tool and BCEPred server is shown in Table 3.

Table 3.B-Cell epitopes of *Ca*. M. haemobos and *M. wenyonii* predicted by immunoinformatics

***Ca.* M. haemobos INIFAP01**			***M. wenyonii* INIFAP02**	
DNA gyrase subunit B			Ribosomal protein SSU S7p	
Position (aa)	Epitope sequence		Position (aa)	Epitope sequence
23-33	SSIRVLEGLEA		8-16	LARRIVYNA
48-59	KGLHHLIWEVLD		35-43	AIRNVAPSI
77-87	LKKGHVISVSD		54-63	NYQVPVESSK
131-138	GVGSTCVN		67-76	EALALRWLIK
140-150	LSSFLEVNVYR		Translation Elongation factor G	
216-225	GKKFVFVNEI			
251-261	SIHDVIFIHSE		Position (aa)	Epitope sequence
289-297	SSIVHSFCN		26-33	ERILYYTG
313-321	DGLLSCIRE		70-77	WKGVVLNL
392-401	QRKVILQRVD		122-132	NKYKVPRIIFC
442-451	FSELYVVEGD		154-161	NIKFSPIQ
546-554	LLEHGYVYI		212-220	LLNEVLVYD
565-574	NKEVVYLFDD		237-244	EIKMCIRK
DNA gyrase subunit A			Translation Elongation factor Thermo unstable (Tu)	
Position (aa)	Epitope sequence		Position (aa)	Epitope sequence
97-105	SIYDALVRM		167-178	NTPVIRGSALKA
107-116	QDFSLRYPLI		Ribosomal protein SSU S12p	
139-148	RLSKLGKYFL			

SSU ribosomal protein S7p	
Position (aa)	Epitope sequence
83-91	SNYQVPVEA
97-106	ETLSLRWLIN
116-125	MVEALAHEII

Translation Elongation factor G	
Position (aa)	Epitope sequence
82-96	GHVDFTVEVERSLRV
142-151	YFKSVQSLRE
143-149	YFKSVQSL
153-162	LKVNAVLIQL
170-178	FIGIIDLIT
211-219	ELLDEVLTY
235-244	VEEIKHCIRI
272-282	VIDYLPSPIDL
387-397	VKGNQVVLESM
420-428	SMVLSRLSE
451-459	ELHLEILID
518-525	EFVDKIVG

Translation Elongation factor Thermo unstable (Tu)	
Position (aa)	Epitope sequence
97-107	AQIDAAILVVS

SSU ribosomal protein S12p	
Position (aa)	Epitope sequence
20-27	KSQALLKS
31-39	LDKKVTFQS
42-49	FKRGVCTR
61-68	ALRKYAKV
72-81	NNYEVLAYIP
87-97	LQEHHVVMVRG

DNA-directed RNA polymerase beta subunit	
Position (aa)	Epitope sequence
61-71	NGLFCESIFGP
111-120	HIELACPVAH
168-177	IFSELEIIDV

DNA-directed RNA polymerase	
Position (aa)	Epitope sequence
7-17	RVNSFSPIVNR
32-42	DLKGVQLGAYT

Position (aa)	Epitope sequence
10-18	LRKFAKVRV
20-29	NNHEVLAYIP
35-42	LQEHHVVM

Ribosomal protein LSU L35p	
Position (aa)	Epitope sequence
15-24	LSKRVVVLGS
59-67	QYKITAHLL

Translation Initiation factor 3	
Position (aa)	Epitope sequence
8-18	RQLDLLLVNNK
20-29	NPPVVKLLNF
84-94	EKVQLIIRTPY

Ribosomal protein LSU L20p	
Position (aa)	Epitope sequence
87-94	FVLNRKVL
107-114	KIVDTSVK

114-124	SVENVFLGNLP			
140-147	FIISQIVR			
205-214	LIQFPLTTLL			
320-327	EIIKVTLE			
468-477	IEELLIYRTH			
532-540	LKLVDELLQ			
587-597	KPFQIVVKEFF			
677-685	FFVTPYYRV			
747-754	CHQLVSVS			
756-763	SLIPFLEH			
849-859	CKNQYPLVRPK			
901-908	IILSSRLI			
966-975	DILVGKVSPL			
1096-1103	QVLETHLG			
1340-1347	CLKINVQY			

Discussion

The hemoplasmas have underwent phylogenetic reclassification after several studies based on molecular markers [33]. Their genome size variation, positional shuffling of genes and poorly conserved gene synteny are evidence of the high dynamic of their genomes [2]. In this work, we found that 12 genomes of hemoplasmas are classified in two groups, and have a different number of CDS and number of tRNAs, additionally, the G+C content vary from 30.46 to 39.27% between the members of the two groups. Thus, G+C content is not specific to each group. Specifically, in bovine hemoplasmas we found differences in genomic features between species. The genome of '*Ca.* M. haemobos' INIFAP01 is significantly longer in length than two genomes of *M. wenyonii* species, but '*Ca.* M. haemobos' INIFAP01 has a lower G+C content. Also, the number of tRNA genes and distribution of rRNA genes are specific to each species. The phylogenetic tree shows that '*Ca.* M. haemobos' INIFAP01 is phylogenetically distant through evolution with *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts. Also, the INIFAP02 and Massachusetts strains are closely related through the evolution of group 2.

The number of genes in the core, soft, shell and cloud genomes revealed in the pan-genomic analysis suggest that there is considerable loss/gain of genes through evolution of the 12 hemoplasmas genomes. Also, the genomes of group 1 (four genomes of three species) are more conserved than group 2 (eight genomes of six species); however, to confirm the previous result it is necessary to use a greater number of genomes of different species of group 1.

In regard to pan-genomic analysis of bovine hemoplasmas, due to the low number of gene clusters in the core genome it confirms that '*Ca.* M. haemobos' INIFAP01 is a divergent species from *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts. In comparative genomics of bovine hemoplasmas, the low percentages of alignment coverage and identity between *Ca*. M. haemobos, *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts in the ANI values suggest that *Ca.* M. haemobos INIFAP01 genome has a different structure than genomes of *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts.

Surprisingly, the alignment coverage and identity percentages among both genomes of *M. wenyonii* (strains INIFAP02 and Massachusetts) suggest that these strains may not belong to the same species because the ANI values were <95%, the species ANI cutoff value [34–36].

A visual evaluation of circular map suggests that genomes of three bovine hemoplasmas are not conserved between them, in fact, this data confirms that '*Ca.* M. haemobos' INIFAP01 genome has a highly different structure than genomes of *M. wenyonii* INIFAP02 and *M. wenyonii* Massachusetts.

Since bovine hemoplasmas show significant differences at genomic level and they impact in cattle health causing economic losses, we decided to perform an immune-informatic analysis to identify B-cell epitopes that could be used in the design of potential vaccines to prevent bovine hemoplasmosis. The development of vaccines based on this strategy has been successfully used

to prevent some diseases in human and animals, including the cytoplasmic protein subolesin used to prevent infestations of tick *Rhipicephalus microplus* [37–40]. The immune-informatics analysis predicts several B-cell epitopes (Table 3), which could be used in the design of molecular detection methods and vaccines. Peptides that contains epitopes have been applied successfully for pathogen detection by serological methods [41,42], immunolocalization of pathogen proteins [43] and vaccines against animal diseases [44–46]. The epitope collection generated in this work will help to design molecular tools that contribute to prevent or diagnose bovine hemoplasmosis.

Conclusions

In this work, we described the main genomic characteristics and the evolutionary relationships between three bovine hemoplasmas. This is the first study to report the genomic characteristics of bovine hemoplasma species. Also, the data presented here about antigenic peptides of *M. wenyonii* INIFAP02 and *Ca.* M. haemobos INIFAP01 identified by immune-informatics have potential uses to detect and/or prevent hemoplasmosis.

Acknowledgements

To Maria Gabriela Guerrero Ruiz (Bioinformatics Analysis Unit, CCG, UNAM) for kindly collaborating with the comparative genomics analysis when using the CIRCOS program. This work was partially granted by Consejo Nacional de Ciencia y Tecnología (CONACyT) Project PN-CONACyT 248855 and CONACyT scholarship 293552.

References

Davidson BL, Allen ED, Kozarsky KF, Wilson JM, and Roessler BJ (1993). A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. Nat Genet 3, 219–223.

References

1. Messick JB. Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Vet Clin Pathol [Internet]. 2004 Mar [cited 2017 Mar 2];33(1):2–13. Available from: http://doi.wiley.com/10.1111/j.1939-165X.2004.tb00342.x
2. Guimaraes AMS, Santos AP, do Nascimento NC, Timenetsky J, Messick JB. Comparative genomics and phylogenomics of hemotrophic mycoplasmas. Balish MF, editor. PLoS One [Internet]. 2014 Mar 18;9(3):e91445. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3958358/
3. Rikihisa Y, Kawahara M, Wen B, Kociba G, Fuerst P, Kawamori F, et al. Western immunoblot analysis of Haemobartonella muris and comparison of 16S rRNA gene sequences of H. muris, H. felis, and Eperythrozoon suis. J Clin Microbiol. 1997 Apr;35(4):823–9.
4. Neimark H, Johansson KE, Rikihisa Y, Tully JG. Proposal to transfer some members of the genera Haemobartonella and Eperythrozoon to the genus Mycoplasma with descriptions of 'Candidatus Mycoplasma haemofelis', 'Candidatus Mycoplasma haemomuris', 'Candidatus Mycoplasma haemosui. Int J Syst Evol Microbiol [Internet]. 2001;51(3):891–9. Available from: http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-51-3-891
5. dos Santos AP, Guimaraes AMS, do Nascimento NC, SanMiguel PJ, Messick JB. Complete genome sequence of Mycoplasma wenyonii strain Massachusetts. J Bacteriol.

2012 Oct;194(19):5458–9.

6. Martínez-Ocampo F, Rodríguez-Camarillo SD, Amaro-Estrada I, Quiroz-Castañeda RE. Draft genome sequence of "Candidatus Mycoplasma haemobos," a hemotropic mycoplasma identified in cattle in Mexico. Genome Announc [Internet]. 2016 Aug 25;4(4):1–2. Available from: http://genomea.asm.org/content/4/4/e00656-16.abstract

7. Quiroz-Castañeda RE, Martínez-Ocampo F, Dantán-González E. Draft Genome Sequence of Mycoplasma wenyonii, a Second Hemotropic Mycoplasma Species Identified in Mexican Bovine Cattle. Microbiol Resour Announc. 2018;7(9):1–2.

8. Hoelzle K, Hofmann-Lehmann R, Hoelzle LE. 'Candidatus Mycoplasma haemobos', a new bovine haemotrophic Mycoplasma species? Vet Microbiol. 2010;144(3–4):525–6.

9. Tagawa M, Matsumoto K, Inokuma H. Molecular detection of Mycoplasma wenyonii and 'Candidatus Mycoplasma haemobos' in cattle in Hokkaido, Japan. Vet Microbiol. 2008;132(1–2):177–80.

10. Niethammer FM, Ade J, Hoelzle LE, Schade B. Hemotrophic mycoplasma in Simmental cattle in Bavaria: Prevalence, blood parameters, and transplacental transmission of 'Candidatus Mycoplasma haemobos' and Mycoplasma wenyonii. Acta Vet Scand. 2018;60(1):1–8.

11. Genova SG, Streeter RN, Velguth KE, Snider TA, Kocan KM, Simpson KM. Severe anemia associated with Mycoplasma wenyonii infection in a mature cow. Can Vet J [Internet]. 2011 Sep;52(9):1018–21. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3157061/

12. Ade J, Niethammer F, Schade B, Schilling T, Hoelzle K, Hoelzle LE. Quantitative analysis of Mycoplasma wenyonii and 'Candidatus Mycoplasma haemobos" infections in

356 cattle using novel gapN-based realtime PCR assays. Vet Microbiol. 2018;220(December
357 2017):1–6.
358 13. Hoelzle K, Winkler M, Kramer MM, Wittenbrink MM, Dieckmann SM, Hoelzle LE.
359 Detection of Candidatus Mycoplasma haemobos in cattle with anaemia. Vet J [Internet].
360 2011 Mar;187(3):408–10. Available from:
361 http://www.sciencedirect.com/science/article/pii/S1090023310000328
362 14. Tagawa M, Yamakawa K, Aoki T, Matsumoto K, Ishii M, Inokuma H. Effect of Chronic
363 Hemoplasma Infection on Cattle Productivity. J Vet Med Sci [Internet]. 2013 Oct
364 15;75(10):1271–5. Available from:
365 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3942926/
366 15. McFadden A, Ha HJ, Donald JJ, Bueno IM, van Andel M, Thompson JC, et al.
367 Investigation of bovine haemoplasmas and their association with anaemia in New Zealand
368 cattle. N Z Vet J. 2016 Jan;64(1):65–8.
369 16. Fujihara Y, Sasaoka F, Suzuki J, Watanabe Y, Fujihara M, OOSHITA K, et al. Prevalence
370 of Hemoplasma Infection among Cattle in the Western Part of Japan. J Vet Med Sci.
371 2011;73(12):1653–5.
372 17. Girotto A, Zangirolamo AF, Bogado ALG, Souza ASLE, da Silva GCF, Garcia JL, et al.
373 Molecular detection and occurrence of ‘Candidatus Mycoplasma haemobos’ in dairy cattle
374 of Southern Brazil. Rev Bras Parasitol Vet. 2012;21(3):342–4.
375 18. Tagawa M, Ybanez AP, Matsumoto K, Yokoyama N, Inokuma H. Prevalence and Risk
376 Factor Analysis of Bovine Hemoplasma Infection by Direct PCR in Eastern Hokkaido,
377 Japan. J Vet Med Sci. 2012;74(9):1171–6.
378 19. Parvizpour S, Pourseif MM, Razmara J, Rafi MA, Omidi Y. Epitope-based vaccine

379 design: a comprehensive overview of bioinformatics approaches. Drug Discov Today
380 [Internet]. 2020; Available from: https://doi.org/10.1016/j.drudis.2020.03.006
381 20. Ahmad TA, Eweida AE, Sheweita SA. B-cell epitope mapping for the design of vaccines
382 and effective diagnostics. Trials Vaccinol [Internet]. 2016;5:71–83. Available from:
383 http://www.sciencedirect.com/science/article/pii/S1879437816300079
384 21. Ranjbar MH, Ebrahimi MM, Shahsavandi MM, Farhadi S, Mirjalili T, Tebianian A, et al.
385 Novel applications of immuno-bioinformatics in vaccine and bio-product developments at
386 research institutes. Arch Razi Inst. 2019;74(3):219–33.
387 22. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: quality assessment tool for
388 genome assemblies. Bioinformatics [Internet]. 2013 Apr 15;29(8):1072–5. Available
389 from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3624806/
390 23. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server:
391 rapid annotations using subsystems technology. BMC Genomics. 2008 Feb;9:75.
392 24. Laslett D, Canback B. ARAGORN, a program to detect tRNA genes and tmRNA genes in
393 nucleotide sequences. Nucleic Acids Res. 2004 Jan;32(1):11–6.
394 25. Lagesen K, Hallin P, Rødland EA, Staerfeldt H-H, Rognes T, Ussery DW. RNAmmer:
395 consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res.
396 2007;35(9):3100–8.
397 26. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search
398 tool. J Mol Biol. 1990 Oct;215(3):403–10.
399 27. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high
400 throughput. Nucleic Acids Res. 2004;32(5):1792–7.
401 28. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics

and high-performance computing Europe PMC Funders Group. Nat Methods. 2012;9(8):772.

29. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010 May;59(3):307–21.

30. Contreras-Moreira B, Vinuesa P. GET_HOMOLOGUES, a versatile software package for scalable and robust microbial pangenome analysis. Appl Environ Microbiol. 2013 Dec;79(24):7696–701.

31. Martin Shumway CA and SLSSKAPALDMS, Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, et al. Versatile and open software for comparing large genomes. Genome Biol. 2004;5(2):12.

32. Connors J, Krzywinski M, Schein J, Gascoyne R, Horsman D, Jones SJ, et al. Circos : An information aesthetic for comparative genomics. Genome Res. 2009;19(604):1639–45.

33. Peters IR, Helps CR, McAuliffe L, Neimark H, Lappin MR, Gruffydd-Jones TJ, et al. RNase P RNA gene (rnpB) phylogeny of hemoplasmas and other mycoplasma species. J Clin Microbiol [Internet]. 2008 May 1;46(5):1873–7. Available from: http://jcm.asm.org/content/46/5/1873.abstract

34. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol. 2007;57(1):81–91.

35. Richter M, Rosselló-Móra R. Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci U S A. 2009;106(45):19126–31.

36. Figueras MJ, Beaz-Hidalgo R, Hossain MJ, Liles MR. Taxonomic affiliation of new

genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. Genome Announc. 2014;2(6):6–7.

37. Michel-Todó L, Reche PA, Bigey P, Pinazo MJ, Gascón J, Alonso-Padilla J. In silico Design of an Epitope-Based Vaccine Ensemble for Chagas Disease. Front Immunol. 2019;10(November):1–16.

38. Hajissa K, Zakaria R, Suppian R, Mohamed Z. Epitope-based vaccine as a universal vaccination strategy against Toxoplasma gondii infection: A mini-review. J Adv Vet Anim Res [Internet]. 2019 Mar 24;6(2):174–82. Available from: https://pubmed.ncbi.nlm.nih.gov/31453188

39. Manzano-Román R, Díaz-Martín V, Oleaga A, Pérez-Sánchez R. Identification of protective linear B-cell epitopes on the subolesin/akirin orthologues of Ornithodoros spp. soft ticks. Vaccine. 2015 Feb;33(8):1046–55.

40. de la Fuente J, Moreno-Cid JA, Canales M, Villar M, de la Lastra JMP, Kocan KM, et al. Targeting arthropod subolesin/akirin for the development of a universal vaccine for control of vector infestations and pathogen transmission. Vet Parasitol. 2011 Sep;181(1):17–22.

41. Liu X, Marrakchi M, Xu D, Dong H, Andreescu S. Biosensors based on modularly designed synthetic peptides for recognition, detection and live/dead differentiation of pathogenic bacteria. Biosens Bioelectron [Internet]. 2016;80:9–16. Available from: http://www.sciencedirect.com/science/article/pii/S0956566316300410

42. Quiroz-Castañeda R, Tapia-Uriza TR, Mujica-Valencia C, Rodríguez-Camarillo SD, Preciado-De la Torre JF, Amaro-Estrada I, et al. Synthetic peptides-based indirect ELISA for the diagnosis of bovine anaplasmosis. Int J Appl Res Vet Med. 2019;17(2):65–70.

448 43. Molina AIB, Bayúgar RC, Gutiérrez-Pabello JA, López ATT, De La Torre JFP, Camarillo
449 SDR. Immunolocalization of VirB11 protein in the Anaplasma marginale outer membrane
450 and its reaction with bovine immune sera. Rev Mex Ciencias Pecu. 2018;9(4):769–91.
451 44. Wang CY, Chang TY, Walfield AM, Ye J, Shen M, Chen SP, et al. Effective synthetic
452 peptide vaccine for foot-and-mouth disease in swine. Vaccine [Internet].
453 2002;20(19):2603–10. Available from:
454 http://www.sciencedirect.com/science/article/pii/S0264410X02001482
455 45. Droppa-Almeida D, Franceschi E, Padilha FF. Immune-Informatic Analysis and Design of
456 Peptide Vaccine From Multi-epitopes Against Corynebacterium pseudotuberculosis.
457 Bioinform Biol Insights [Internet]. 2018;12:1177932218755337. Available from:
458 https://europepmc.org/articles/PMC5954444
459 46. Bashir S, Abd-elrahman KA, Hassan MA, Almofti YA. Multi Epitope Based Peptide
460 Vaccine against Mareks Disease Virus Serotype 1 Glycoprotein H and B. Am J Microbiol
461 Res. 2018;6:124–39.

462 Supporting information

463 **S1_Fig. Pan-genomic analysis of 12 hemoplasmas.**

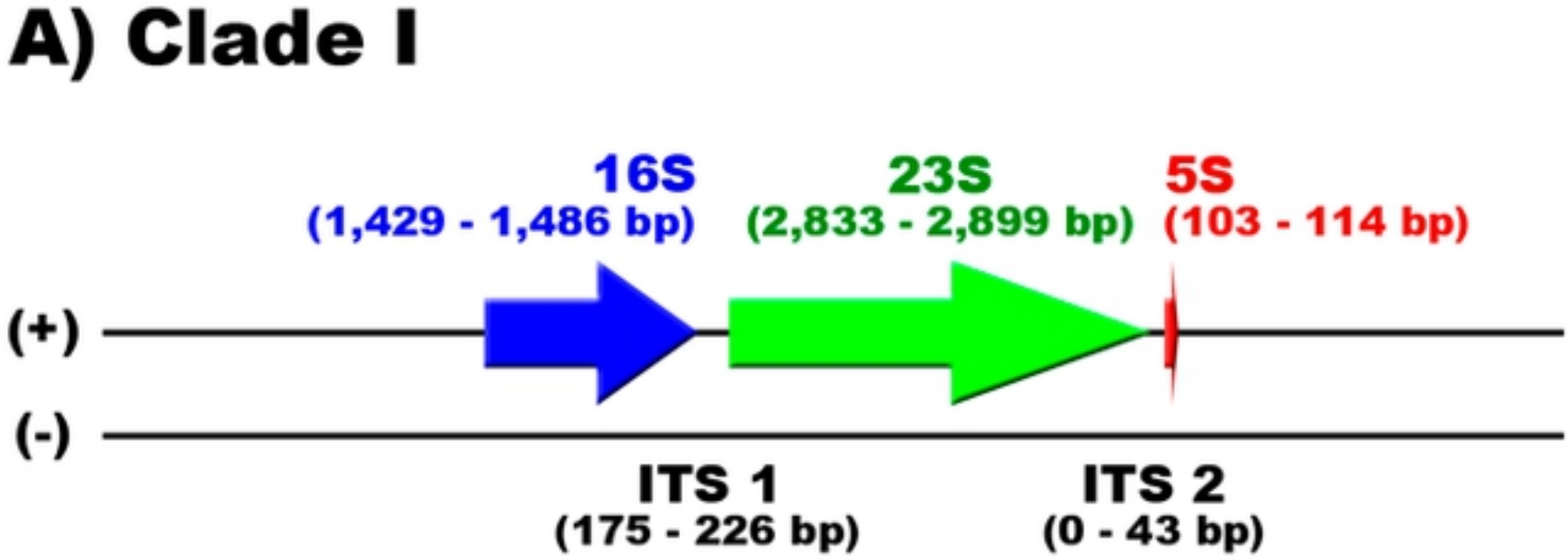
464 **S2_Fig. Core, soft core, shell and cloud genomes of hemoplasmas.**

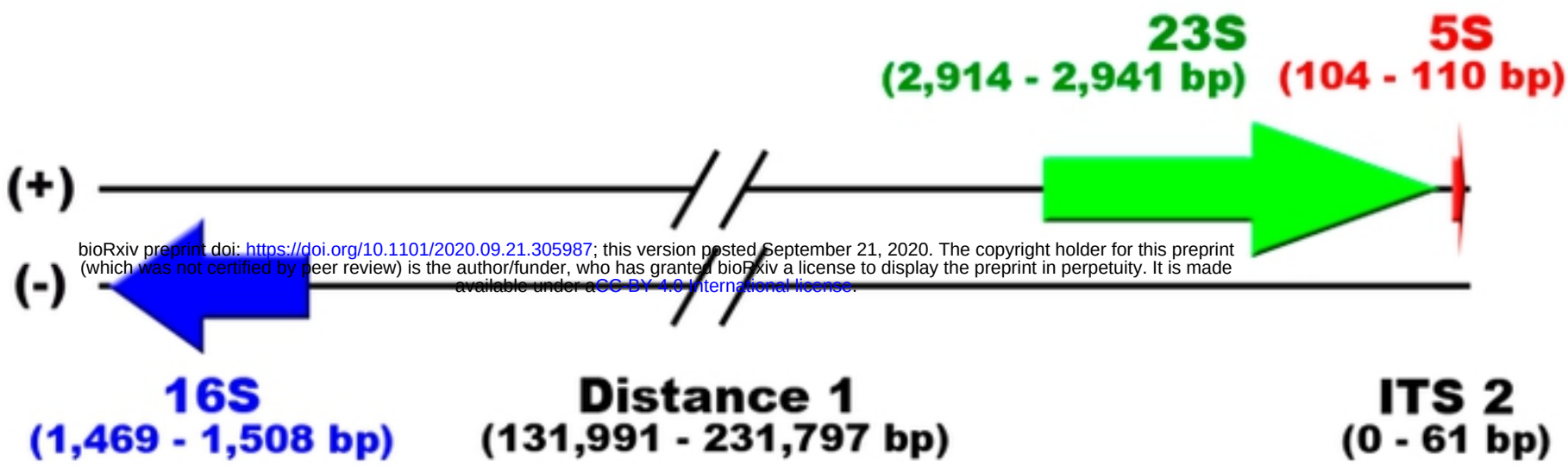
465 **S1_Table. 12 hemoplasma genomes reported in the GenBank database.**

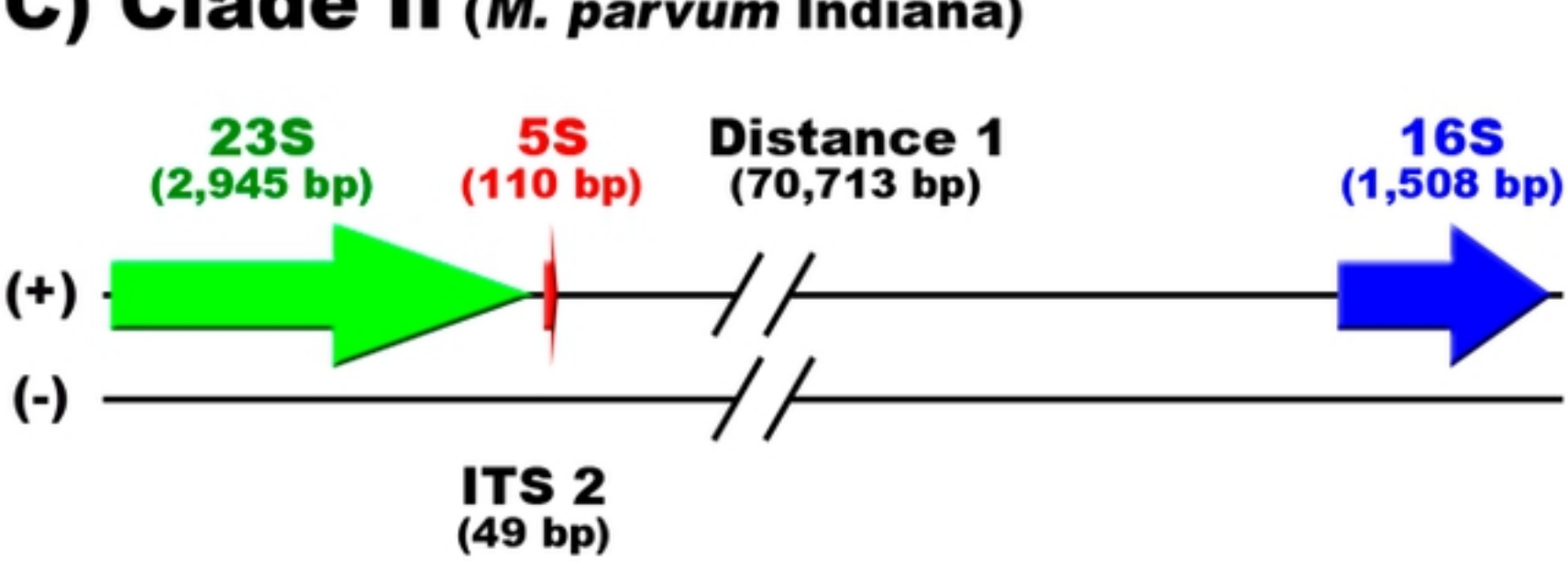
466 **S2_Table. BLAST-based average nucleotide identity (ANIb) values of alignment coverage**
467 **of 12 hemoplasma genomes.**

468 **S3_Table. BLAST-based average nucleotide identity (ANIb) values of identity of 12**
469 **hemoplasma genomes.**

470







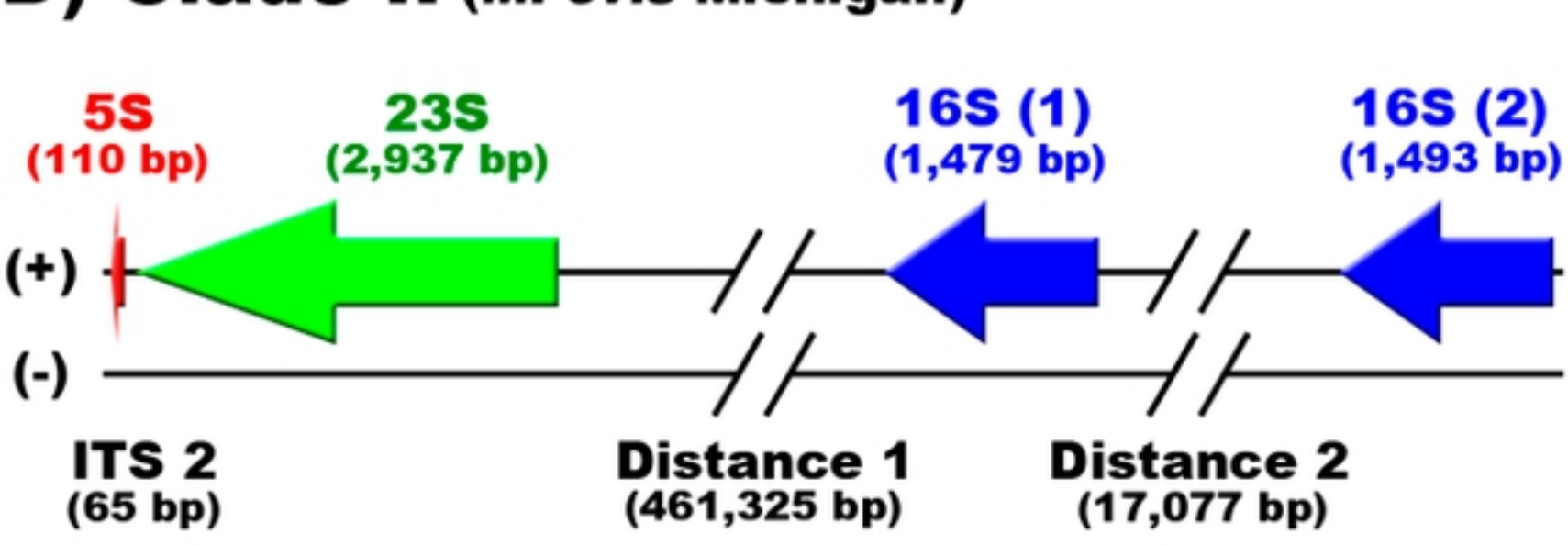
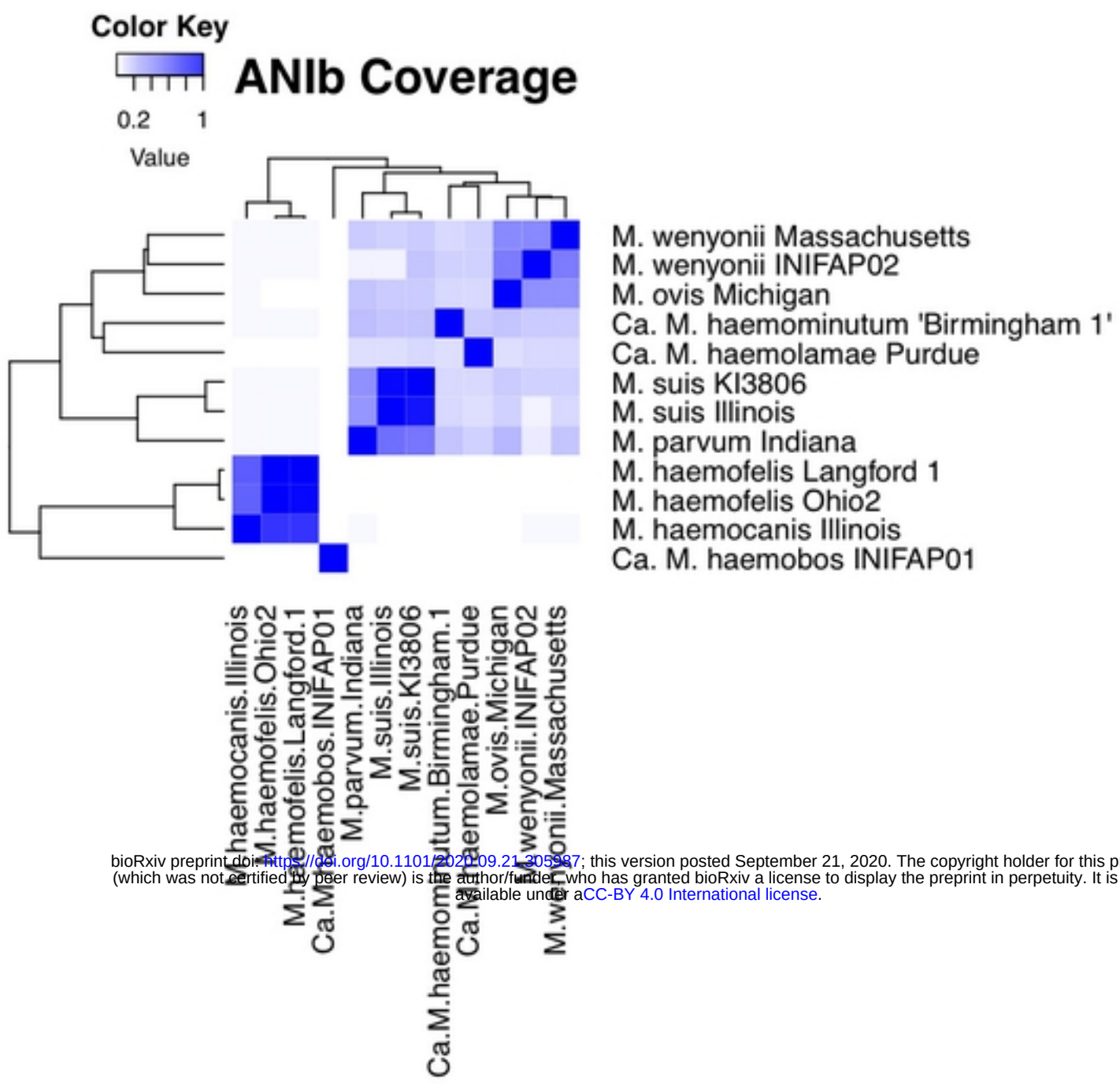


Fig1

A)



B)

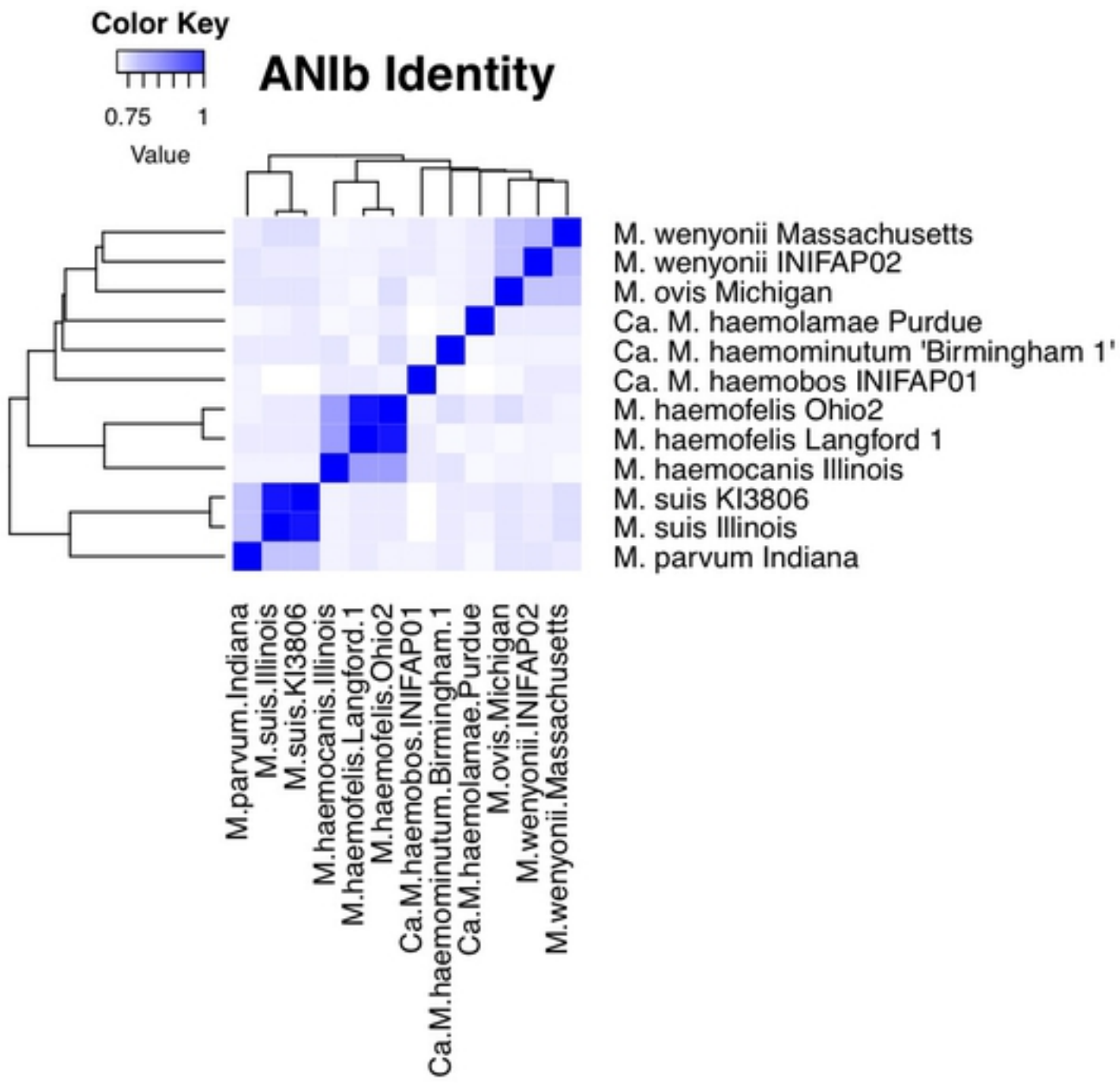


Fig3

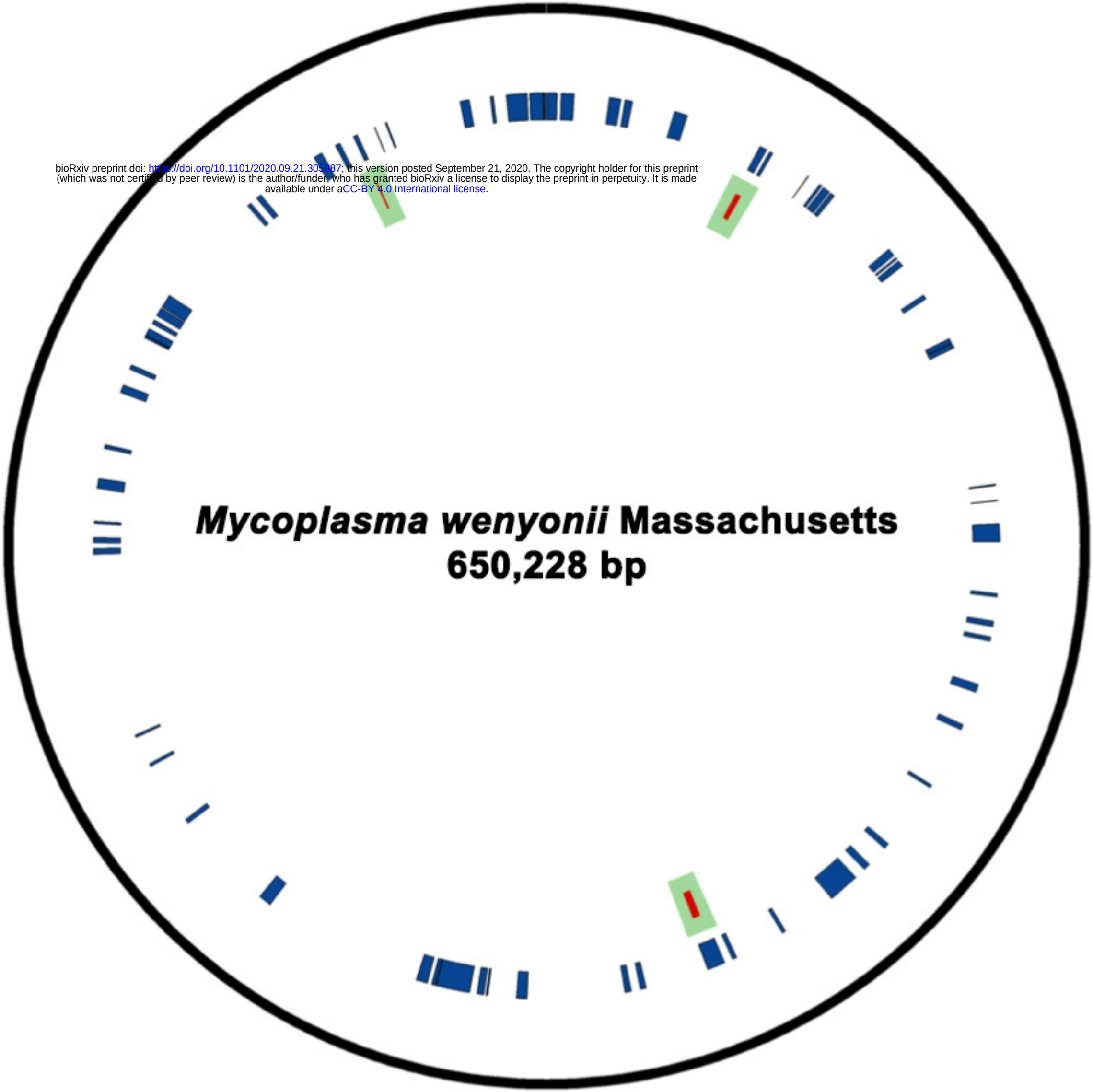


Fig4

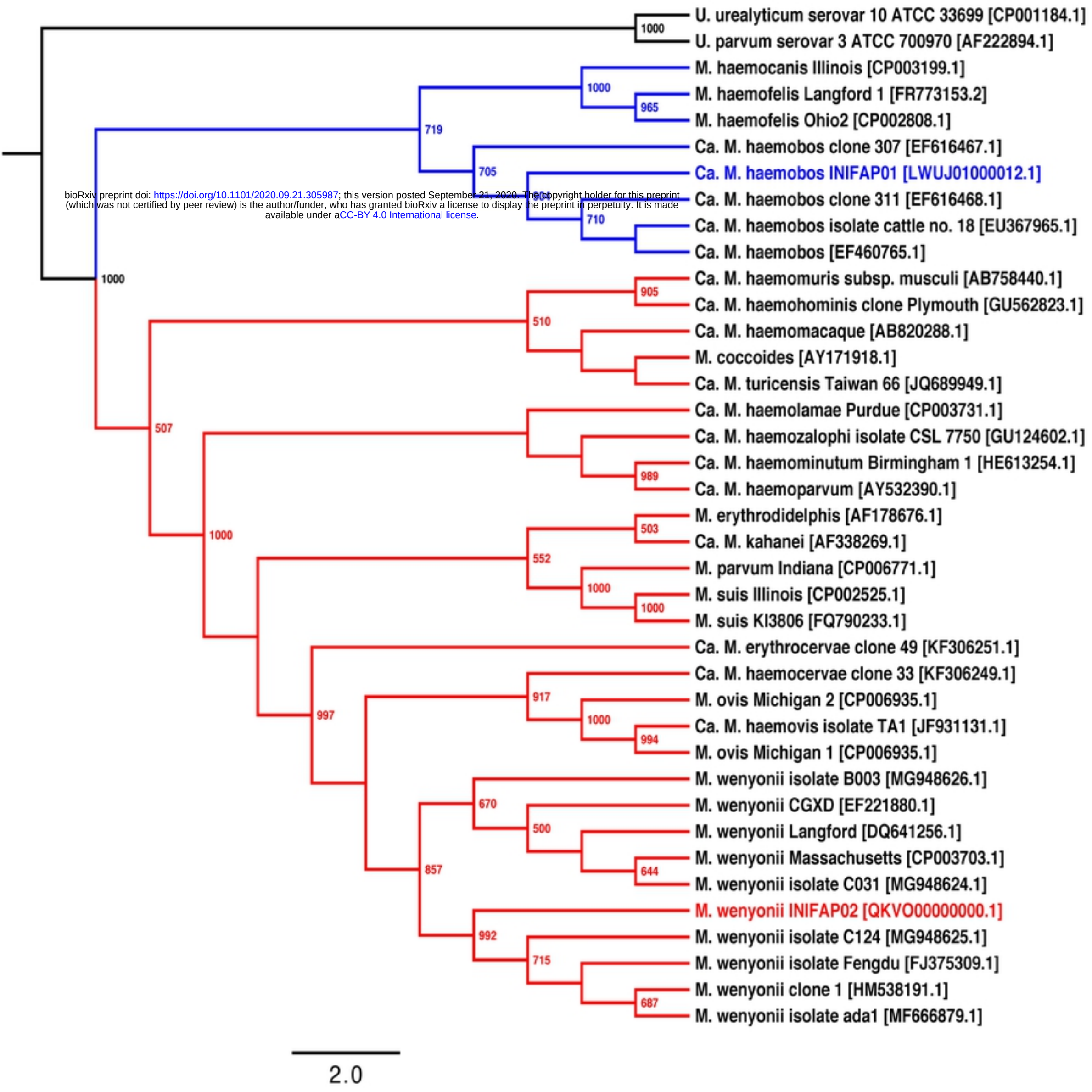


Fig2