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Genome erosion and evidence for an intracellular niche – exploring the biology of mycoplasmas in Atlantic salmon

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A B S T R A C T

Mycoplasmas are the smallest autonomously self-replicating life form on the planet. Members of this bacterial genus are known to parasitise a wide array of metazoans including vertebrates. Whilst much research has been significant targeted at parasitic mammalian mycoplasmas, very little is known about their role in other vertebrates. In the current study, we aim to explore the biology of mycoplasmas in Atlantic Salmon, a species of major significance for aquaculture, including cellular niche, genome size structure and gene content. Using fluorescent *in-situ* hybridisation (FISH), mycoplasmas were targeted in epithelial tissues across the digestive tract (stomach, pyloric caecum and midgut) from different development stages (eggs, parr, subadult) of farmed Atlantic salmon (*Salmo salar*), and we present evidence for an intracellular niche for some of the microbes visualised. *Via* shotgun metagenomic sequencing, a nearly complete, albeit small, genome (~0.57 MB) as assembled from a farmed Atlantic salmon subadult. Phylogenetic analysis of the recovered genome revealed taxonomic proximity to other salmon derived mycoplasmas, as well as to the human pathogen *Mycoplasma penetrans* (~1.36 Mb). We annotated coding sequences and identified riboflavin pathway encoding genes and sugar transporters, the former potentially consistent with micronutrient provisioning in salmonid development. Our study provides insights into mucosal adherence, the cellular niche and gene catalog of *Mycoplasma* in the gut ecosystem of the Atlantic salmon, suggesting a high dependency of this minimalist bacterium on its host. Further study is required to explore and functional role of Mycoplasma in the nutrition and development of its salmonid host.

1. Introduction

Mycoplasmas are a diverse group of bacteria known to parasitise a wide array of metazoans, plants, invertebrates and vertebrates, including fisheries (Razin, 1992). *Mycoplasma* had been isolated from multiple fish species, (Carp: *Cyprinus carpio;* Tench *Tinca tinca*; Trout: *Salmo trutta*; Eel: *Anguilla anguilla;* Sheat fish: *Silurus glanis*; Mosaic threadfin: *Trichogaster leeri*; cichlid:*Tropheus sp.*; Plaice: *Pleuronectes platessa*; Salmon: *Salmo salar*; goldfish: *Carassius aurarus*; Brook lamprey: *Lampetra planeri*) by the early 1980s (Kirchhoff and Rosengarten, 1984). More recently, several studies have identified *Mycoplasma* from marine teleosts using culture-free approaches. Mudsucker (*Gillichthys mirabilis*) and pinfish (*Lagodon rhomboids),* for example, have been identified as having gut microbiomes rich in *Mycoplasma* (Egerton et al., 2018). However, salmonids in particular are frequently reported to be colonised by *Mycoplasma* (Holben et al., 2002; Llewellyn et al., 2016). This is especially true in Atlantic salmon (*Salmo salar*), both in wild and in farmed settings (Holben et al., 2002; Zarkasi et al., 2016). In some cases, *Mycoplasma* phylotypes can comprise >70% of the total microbial reads recovered from salmon intestines (Heys et al., 2020; Llewellyn et al., 2016). The distribution and biological role of *Mycoplasma* in the intestines of salmonids are far from clear and require further exploration. Nonetheless, demographic modelling of microbial communities suggests colonisation of salmonid guts by these microorganisms as *non-neutral*, *i.e.* the rate at which these bacteria colonise the gut indicates a significant degree of specific adaptation to the host environment (Cheaib et al., 2020; Heys et al., 2020). Interestingly, Mycoplasma sp. are also prevalent in different extraintestinal organs (gills, liver, spleen, kidney, reproductive organs, serous membrane from the peritoneum and the swim bladder) of different fish species (EI-Jakee Ei-Jakee, 2020; Sellyei et al., 2021). Even after 40 years form their discovery, the role of Mycoplasma sp. in fisheries and aquaculture production is not clear. In particular, questions around Mycoplasma pathogenicity remains unresolved.

Mycoplasmas, as well as related taxa included in the class Mollicutes (*Spiroplasmas*, *Ureaplasma* and *Acholeplasmas*), are recognized as the

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smallest and simplest free-living and self-replicating forms of life (Bové, 1993; Trachtenberg, 2005). Mycoplasmas lack a peptidoglycan cell wall and are bounded by a simple cell membrane (Miyata and Ogaki, 2006). In addition to being physically small, mycoplasmas have the smallest genomes of any free-living organism (Razin et al., 1998). *Mycoplasma genitalium*, in particular, has a genome size of 580 kilobases comprising of only 482 protein-coding genes(Citti and Blanchard, 2013), whilst *Mycoplasma mycoides*, typically has 473 protein-coding genes, of which 149 still have no known function (Citti and Blanchard, 2013).

The simplicity of mycoplasmas, their small genome sizes, as well as their close association with metazoan hosts has led them to be considered as a target species to explore theories around genome erosion or reductive evolution (Fadiel et al., 2007; Rocha and Blanchard, 2002). Dependence on host organisms can theoretically lead to mutual interdependence of metabolic processes. This results in its relaxed selection among the pool of bacterial genomes, with the main process being the accumulation of loss-of-function mutations in coding genes, and the eventual loss of genetic material from the bacterial genome (Boscaro et al., 2017). Genetic drift can also play a significant role as host-associated microbes have relatively fewer opportunities to exchange genetic material with the wider microbial community (Moran, 1996). Isolation from microbial congeners and host dependence may be further enhanced in mycoplasmas that exploit an intracellular niche, which several species have been shown to do within the literature (Razin et al., 1998; Yavlovich et al., 2004). Mycoplasmas, likely owing to their dependence on their hosts, have fastidious requirements for *in vitro* culture. Culture-free approaches for microbial identification, especially, with the advent of DNA sequencing approaches, have markedly increased in recent years to identify new *Mycoplasma* strains (Aceves et al., 2018; Costello et al., 2013; Martin et al., 2013).

In the current study, we aimed to explore the characteristics of *Mycoplasma* in salmonids, including a potentially intracellular niche, taxonomic affiliations, genome structure and gene content. We also examined genetic features and metabolic functions that reveal the role of reductive evolution in shaping its genome. Finally, we discuss what role *Mycoplasma* may have in impacting host fitness, especially in the context of aquaculture, by reviewing genomic features it possesses that are consistent with parasitic or symbiotic lifestyles.

2. Materials and methods

2.1. Sample collection

Farmed *Atlantic salmon* (*Salmo salar*) subadults (individuals from 3 to 5 kg) fed on a commercial diet were sampled from 3 marine cages at an aquaculture facility at Corran Ferry, near Fort William, Scotland, in Autumn 2017 in collaborations with MOWI Ltd. *Salmo salar* freshwater parr (30-50 g) and ova were sampled at the Institute of Biodiversity, Animal Health and Comparative Medicine aquarium facility, University of Glasgow. The fishes were euthanized by blunt cranial trauma under a Schedule 1 procedure and gut compartments (stomach, pyloric caecum, and midgut) samples were dissected under aseptic conditions before being fixed in formalin for subsequent microscopy, or flash frozen in liquid nitrogen prior to DNA analysis.

2.2. Fluorescence in-situ hybridisation (FISH)

Previous work has established the dominance of *Mycoplasma* in marine *Salmo salar* GI (gastrointestinal tract) (Heys et al., 2020). To explore their physical distribution in different gut compartments and life cycle stages, FISH was undertaken on stomach, pyloric caecum and hind gut from marine farmed adults (three individuals, MOWI, Scotland) and juvenile parr reared in aquaria at the University of Glasgow (three individuals). Samples were fixed in a freshly made sterile-filtered solution of 4% paraformaldehyde in PBS (pH 7.4) for 16–24 h and maintained at room temperature for 16–48 h. Fixed samples were then washed with sterile-filtered PBS (pH 7.4) three times before being stored in 70% ethanol. Samples were then gradually dehydrated in a series of ethanol-xylene-paraffin treatment steps (Copper et al., 2018). Before sectioning, samples were embedded in paraffin and stored at 4 °C. At least four 3–4 μm sections were taken from each embedded tissue block, rehydrated in sterile ddH_20, and mounted on slides for pepsin treatment and straining. Pepsin treatment was undertaken in a 0.05% pepsin solution and 0.01 M HCL. Samples were DAPI stained to target cell nuclei of host cells, and FISH probes were hybridized at 55 °C to the 16S rDNA small subunit of bacterial cells. Multiple FISH probes labelled with Cy3 and Cy5 dyes were deployed to distinguish *Mycoplasma* strains from other microbes present in samples (Table 1). To improve the visualization of non-mycoplasma bacteria, multiple probes were deployed using the same dye. A *Mycoplasma* probe (Myc1–1) (Table 1) probe was designed based on Illumina amplicon sequences based upon the most abundant operational taxonomic (OTU) sequence identified in Adult Salmon that we identified in previous work (Heys et al., 2020). To establish respective specificity of probes, positive controls of for both universal probes (*E. coli*) and *Mycoplasma* probes (*Mycoplasma muris)* were used. Attempts culture Mycoplasma from salmon were not successful. All samples were visualised at 20-30× magnification on a DeltaVision-Core microscope (Applied Precision, GE), equipped with a CoolSNAP HQ camera (Photometrics) and operated with SoftWoRx software (Applied Precision, GE).

2.3. DNA extraction, library annotation and sequencing

DNA was extracted from a section of pyloric caecum derived from a single individual on which FISH analyses had identified the presence of *Mycoplasma* organisms, based on their labelling with a targeted 16S probe. The sample homogenised *via* bead beating and DNA extracted using a Qiagen DNAeasy Stool Kit. A sequencing library for Illumina Next-Seq WGS (whole genome shotgun) was prepared using a sonication protocol and a TruSeq library protocol and adaptors. Sequencing was undertaken at the University of Glasgow Polyomics facility.

2.4. Data preprocessing, assembly, binning and annotations

The short paired-end NextSeq Illumina reads (2 × 63 million reads) were preprocessed for quality filtering using sickle V1.2 (https://github.com/najoshi/sickle). Decontamination of good quality reads was performed by mapping reads against the *Salmo salar* genome (available at NCBI sequence archive with the accession number GCF_000233375.1) using Deconseq V 0.4.3 (Schmieder and Edwards, 2011) based on BWA mapper V 0.5.9 (Li, 2013). The decontaminated paired-end reads (~18 millions of bacterial reads) were assembled using the Megahit V1.1 software (Li et al., 2015). The assembled contigs (~93,400) were processed for binning using MetaBAT V2.12.1 (Kang et al., 2015). Quality assessment for completeness and contamination of sequence was performed using CheckM V1.0.18 software (Parks et al., 2015). Annotation of gene content was performed using the pipeline ATLAS-metagenome (Kieser, 2019), which involves the prediction of open reading frames (ORFs) using Prodigal (Hyatt et al., 2010). Translated gene products were clustered using LinClust (Steinegger and Söding, 2018) to generate non-redundant gene and protein catalogues, which were mapped to the eggNOG catalog (Huerta-Cepas et al., 2019) using DIAMOND (Buchfink et al., 2015).

2.5. Phylogenetic analyses

Two approaches were undertaken to construct phylogenetic trees: a) MLST-based (Multi Locus Sequence Typing); and b) 16S gene markers (recovered from the genome) of the *mycoplasma* MAG (metagenome-assembled genome) from this study as well as what is previously available in the literature. Using CheckM software, the MLST-based strategy focused on a concatenation of 21 conserved housekeeping genes

Table 1
Fish probes and sequences deployed in this study.

Probes	Target group	Sequence (5′-3′)	Reference
Myc1–1	*Mycoplasma*	GCGGTAATACATAGGTYGCAAGCG	This study
Gam-1	Gammaproteobacteria	GCCTTCCCACATCGTTT	Manz *et al.*, 1992
FIR-1	Firmicutes	GGAAGATTCCCTACTGCTG	Hallberg *et al.*, 2006
EUB338	All bacteria	GCTGCCTCCCGTAGGAGT	Amann *et al.*, 1990
EUB338 II	*Planctomycetes*	GCAGCCACCCGTAGGTGT	Daims *et al.*, 1999
EUB338 III	*Verrucomicrobia*	GCTGCCACCCGTAGGTGT	
Non EUB338	None	CGACGGAGGGCATCCTCA	Wallner *et al.*, 1993

This table resumes the probes used for targeting general and specific bacterial groups including the Mycoplasmas and control negative.

annotated in the mycoplasma MAG supplemented with the orthologues available for all the *Mycoplasma* genera, to date. The MLST-based dataset included 55 orthologues of protein sequences of concatenated 21 conserved markers. The 16S rDNA sequence dataset included: one sequence of 16S rDNA gene annotated from the mycoplasma MAG; five Operational Taxonomic Units (OTUs) sequences of mycoplasmas characterised form the same farmed salmon system (Heys et al., 2020); 101 and 17 sequences of 16S rDNA from the *Mycoplasma* sp. and *Sprioplasma* sp. genomes respectively from IMG database; and 11 sequences, from environmental studies, detected in marine species including shrimp, fish and isopods. DNA and protein sequences were aligned using MAFFT version 6.24 (Katoh and Standley, 2013). Phylogenetic inference was performed using PhyML version 3.0 (Guindon and Gascuel, 2003) and MrBayes V.3.2.6 (Huelsenbeck and Ronquist, 2001). The evolutionary model was chosen using MODELTEST(Posada and Crandall, 1998), and parameters were iteratively estimated in PhyML using the GTR + I + G model for the nucleotide sequence of 16 s trees and the LG + I + G model for amino-acid sequences of concatenated markers trees (Le and Gascuel, 2008). Bootstrap values were calculated using 100 replicates (Felsenstein, 1985). With MrBayes, posterior probability values were calculated using an average standard deviation of partition frequencies <0.01 as a convergence diagnostic (Ronquist et al., 2012). MrBayes runs consisted of eight simultaneous Markov chains, each with 1,000,000 generations, a subsampling frequency of 1000, and a burn-in fraction of 0.15. Trees were then visualised and adapted for presentation in FigTree version1.4.3 as a graphical viewer of phylogenetic trees (http://tree.bio.ed.ac.uk).

2.6. Metabolic pathways comparison and genome reduction analysis

All Pfam V.32 (comprehensive and accurate collection of protein domains and families) annotations were predicted with Prodigal and analysed in terms of function categories and metabolic content (focusing on Enzyme EC numbers). The 570 genes identified were associated with 746 Pfam functions. The Pfam functions led to the recovery of Gene Ontology (GO) terms and were then mapped to the KEGG database. Simultaneously, the alternate approach involving the MetaCyc database was employed to elucidate metabolic pathways from all domains of life (Caspi et al., 2018). The EC numbers of the coding sequence regions in *Mycoplasma penetrans* were extracted from the KEGG database and were then compared with those annotated within the mycoplasma MAG from *Salmo salar* in this study. The mapping of metabolic pathways from both genomes was visualised using the iPath (Yamada et al., 2011). From the IMG genomic database, all available metadata on sequenced mycoplasma strains were then collected and compared to the mycoplasma MAG for the genome size, GC content, gene content and their preference (*e.g.* intracellular, free-living etc). Annotations for the mycoplasma MAG were submitted to CG view (Grant and Stothard, 2008) for radial visualization of its genomic. Using a core gene approach, the 570 predicted genes were compared at the DNA and protein sequence levels against all the available genes of *Mycoplasma penetrans* using BLAST+ V 2.8.1 (Altschul et al., 1990). The best hits for each query were represented in a radial plot using Circoletto software version V.069–9 (Darzentas, 2010). Complimentary annotations were performed using RAST software which, consisted of subsystem classification of microbial functions available in the curated database, *i.e.* SEED subsystems (Overbeek et al., 2014).

3. Results

3.1. Fluorescence in situ hybridization (FISH) of mycoplasmas in the farmed salmon

The set of probes used in FISH for the identification of bacterial populations is summarized in Table 1. The *Mycoplasma* probe Myc1–1 showed specific hybridization, and its specificity was evaluated against in pure culture *Escherichia coli* and *Mycoplasma muris* (Supplementary Fig. S1). The probe gives a positive signal solely with cultured *Mycoplasma muris*. We made multiple attempts in both solid and liquid culture mycoplasmas from the salmon intestines, but without success. FISH visualization in salmon ova demonstrated a low abundance of bacteria and no signal of *Mycoplasma* sp. (Fig. 1A; Supplementary Fig. S2.1). In *Salmo salar* freshwater parr, *Mycoplasma* sp. aggregates were observed on the stomach lining of all samples (Fig. 1B; Supplementary Fig. S2.2), as well as on the muscular mucosae, and epithelium of the pyloric caecum (Fig. 1C; Supplementary Fig. S2.3). In the midgut of salmon parr (distal to the pyloric caecum) the aggregation of *Mycoplasma* sp. in the epithelium cells was also identified (Supplementary Fig. S2.4). In the stomach (Fig. 1D; Supplementary Fig. S2.5) and pyloric caecum (Fig. 1E–F; Supplementary Fig. S2.6) of adult salmon, *Mycoplasma* sp. signals were clustered in small aggregates in the lumen around the nuclei of epithelial cells. Fig. 1 indicates this intracellular clustering most clearly. In the midgut of adult salmon, *Mycoplasma* sp. showed lower abundance and the signals of *Mycoplasma* sp. showed aggregations near epithelium cell nuclei (Supplementary Fig. S2.7). More comprehensive, 16S -amplicon-seq based surveys of Mycoplsama abundances in farmed sub adults from the same site can be found in Heys et al., 2020.

3.2. Mycoplasma MAG (metagenome-assembled genome) features and orthologs

Using a total of 63,180,207 reads, and after decontamination, 93,397 contigs were assembled using megahit software (see Materials and methods). The assembled contigs were binned, annotated, and assessed for completeness (see materials and methods). The best quality assembled bins corresponded to a nearly complete genome assigned to a *Mycoplasma sp.* (see the genome sequence in Supplementary File 2). The completeness of this metagenome-assembled genome (MAG) was estimated at 92.18% with 0.38% of contamination (Table 2). The metagenome and MAG were deposited into the NCBI database under the Bio project accession number PRJNA714611.

The average size of the assembled genome was estimated to be 0.57 Mb and comprised a set of 570 predicted genes accounting for a total of 694 CDS regions found on the 5′3’ and 3′5’ ORFs. The GC percentage was estimated to be 24.98% (Table 2). Circular representation of the genomic structure of the mycoplasma MAG highlights CDS annotations on the negative (Fig. S3-a) and positive (Fig. S3-b) strands, respectively. To further resolve CDS annotations, a supplementary annotation

framework was applied using the curated SEED database and the RAST server (Glass et al., 2010). The number of curated annotations was reduced to 600 CDS across the negative (275 CDS) and positive strands (325 CDS). Among these CDS regions, 390 had functional annotations, and within these, three annotated CDS regions (> 85% of similarity threshold against SEED) were identified as riboflavin kinase (EC 2.7.1.26/EC 2.7.1.26;1278 bp) along with two Riboflavin/purine transporters of length 1383 bp and 1608 bp, respectively. Other functions required for host-microbiota symbioses, such as ribonucleotide reductase, were annotated with SEED and are reported (Supplementary Table 1).

3.3. Phylogenetic proximity to Mycoplasma penetrans

Robust phylogenetic trees were recovered based on 16S rDNA and MLST data. The 16S rDNA tree includes four OTUs of *Mycoplasma* detected in the digestive tract of farmed salmon previously (Jin et al., 2019), as well as all OTUs recovered from our recent work on the same farm system (Heys et al., 2020). All *Mycoplasma* OTUs, including the MAG we sequenced lie in a clade alongside *M. muris* and *M. penetrans*. (Fig. 2). No evidence of an ecological association between mycoplasmas from similar ecotopes (*e.g.* marine, freshwater, terrestrial) was noted.

To further ascertain the above clustering of 16S rDNA sequences of *Mycoplasma*, and the phylogenetic relatedness, a second analysis based on MLST approach using 21 concatenated housekeeping genes (see PFAM IDs of markers and their functions in Supplementary File 1) increased our confidence in *M. penetrans* being close to the recovered mycoplasma MAG (Fig. 3). These 21 markers are detected in single copies and are conserved in the bacteria and the mycoplasmas lineage. The MLST tree shows high posterior probabilities in support of this topology (post prob. >0.9). Tip labels of the selected *Mycoplasma* sp. were

Table 2
Summary of *Mycoplasma* metagenome assembled genome (MAG).

Genome	*Mycoplasma* assembled genome
Completeness	92.18
Contamination	0.38
Unique markers (of 43)	39
Multi-copy	0
Taxonomy (contained)	Genus: *Mycoplasma*
Taxonomy of sister lineage	*Mycoplasma penetrans*
GC content	24.98
Genome size (mbp)	0.57
Gene count	570
Coding density	0.93
Length	577,903
N50	14,796
Genome completeness	92.18%

The shotgun metagenomics data (63 million reads) were trimmed after a quality control assessment, then assembled using Megahit, binned using Metabat and checked for binning quality using CheckM software. The metagenome was sequenced from the pyloric caecum of one individual subadult farmed Atlantic salmon (*Salmo salar*).

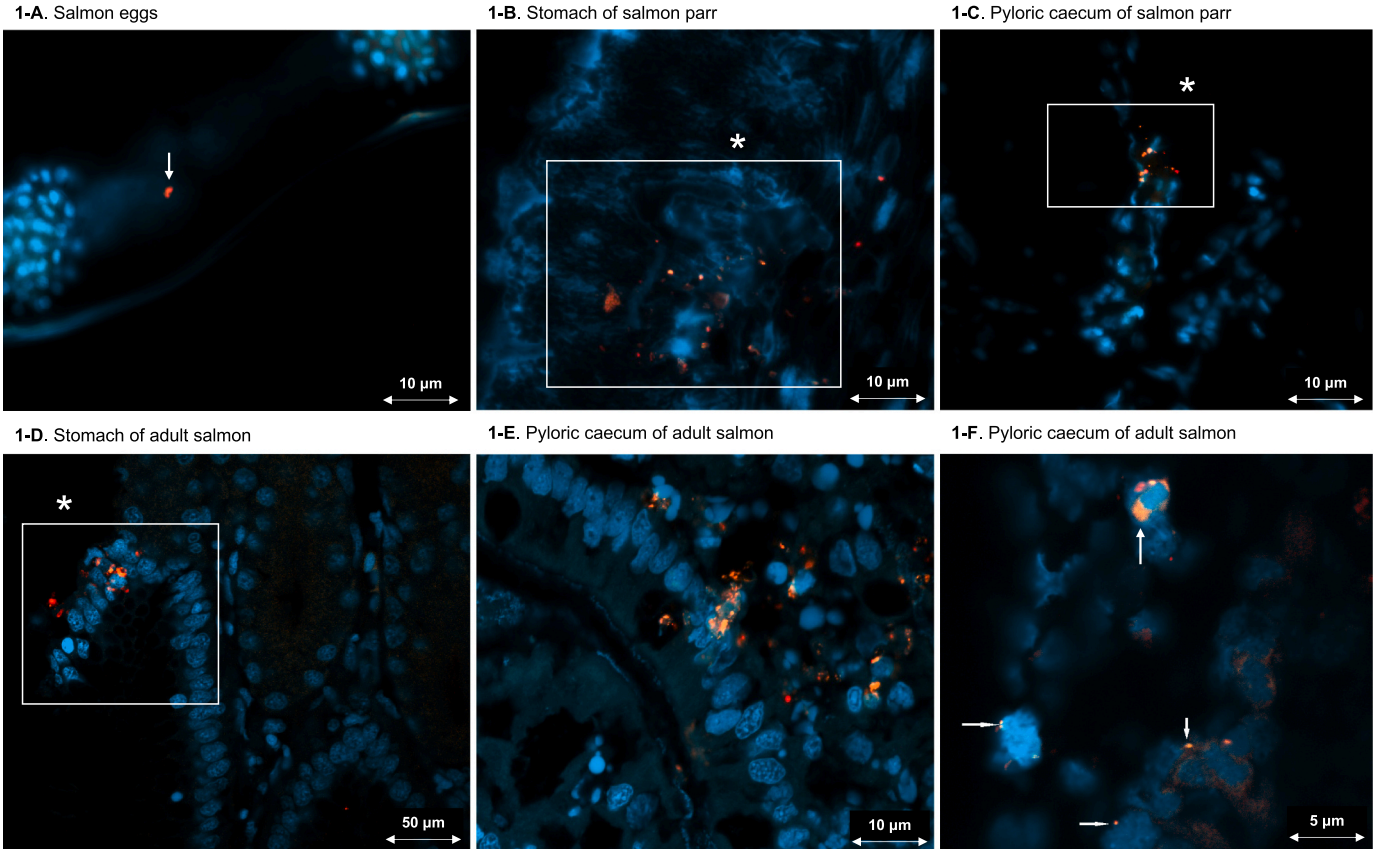

Fig. 1. FISH visualization of Mycoplasma in salmon parr and adults. The images were an overlay of DAPI signals (blue), hybridization signals of Gam-1, FIR-1, EUB338, EUB338 II, EUB338 III probes (Cy5, red) and *Mycoplasma* sp. Specific Myc1–1 probe (Cy3, orange). Scale bars are shown in the bottom left corner of each image (A) *Mycoplasma* sp. are absent from salmon ova, and bacteria scarce. (Scale 10um). (B) Distribution of *Mycoplasma* sp. in the stomach of salmon parr, scaled at 10 µm. Orange signals indicate mycoplasmas were clustered in small groups. (C) Distribution of *Mycoplasma sp.* in the epithelium of pyloric caecum of salmon parr (Scale 10um). (D) Distribution of *Mycoplasma sp.* in the stomach of adult salmon (Scaled 50 µm). (E, F) Distribution of *Mycoplasma sp.* in the pyloric caecum of adult salmon scaled at 10 µm (E) and 5 µm (F) respectively. Mycoplasmas signals were aggregated on the muscularis mucosae, lamina propria (E) and clustered in high abundance around epithelial cell nuclei (white arrows, F). These experiments were performed using at least three technical replicates of each life stage and digestive tract compartment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

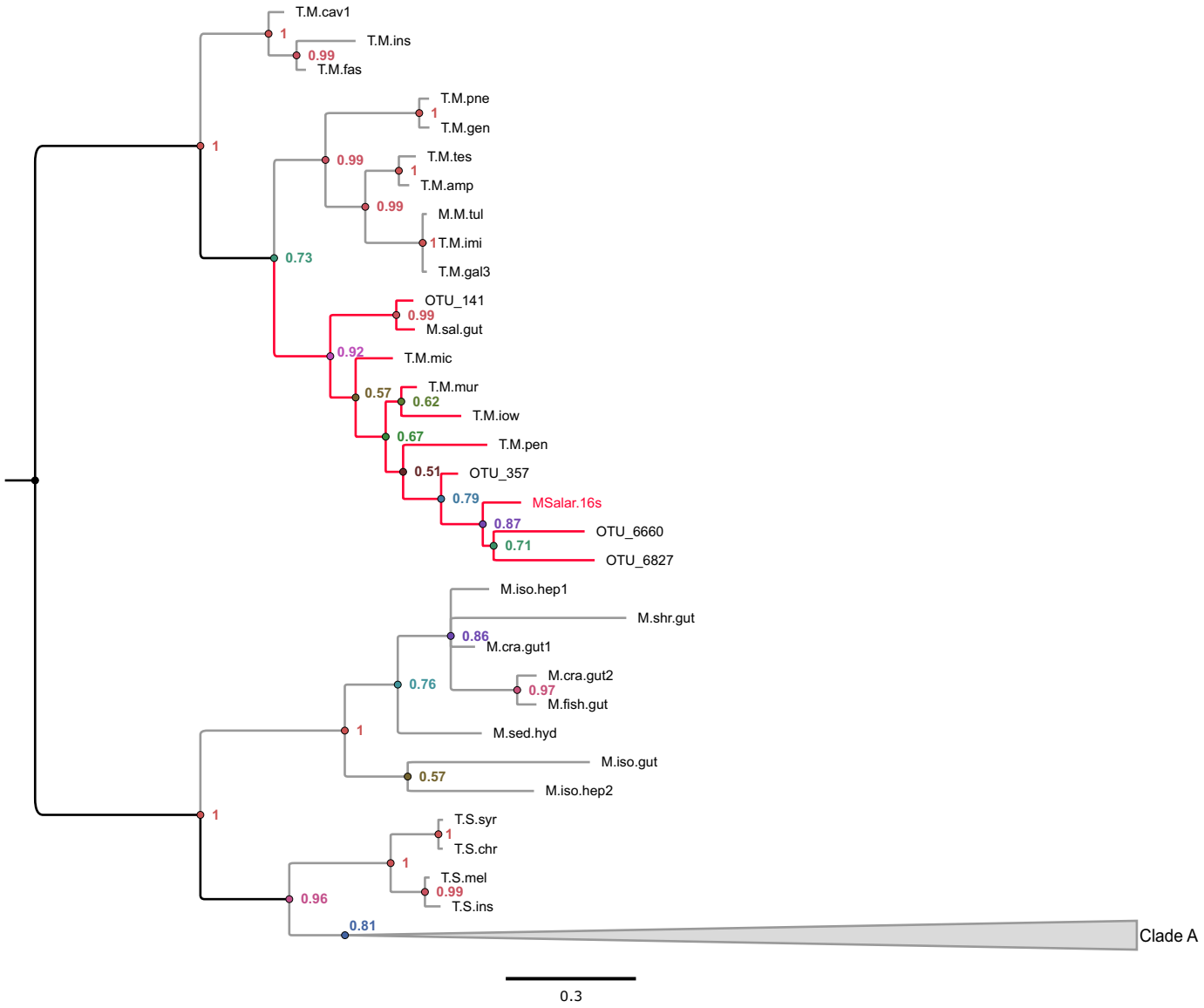

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of mycoplasmas. Sequence name abbreviation of tree tips labels and clade A (including Spiroplasmas) are reported in Supp. File (sheet 2). The tree as consrtucted using MrBayes software , the nodes labels represent the calculated posterior probability values (see Materials and methods).

further annotated with the information of genome size in Mbp. It should be noted that the genome sizes did not appear to correlate with the phylogenetic distribution of *Mycoplasma* sp. in the tree. The genome size of *M. penetrans* (1.36 Mb) is approximately double to that of the mycoplasma MAG and, is the highest among the *Mycoplasma* sp. genomes.

3.4. Orthology, metabolic pathways and genome reduction analyses

A core genome analysis including the amino acid sequences of predicted CDS and all the available CDS from the closely related *M. penetrans*, available on NCBI repository, were blasted against the COG database. Circular track of the genome including the orthology clearly showed the difference in genome size between the mycoplasma MAG and *M. penetrans*. We observed heterogeneity across in terms of GC content and GC skew (Fig. 4). An orthology analysis based on SEED annotations indicated 14 functions (oxidative stress, periplasmic stress, protein biosynthesis, detoxification, ribonuclease H, cation transporters, ABC transporters) specific to the mycoplasma MAG, 144 functions specific to *M. penetrans*, and 156 functions that are common to mycoplasma MAG and *M. penetrans*. The shared functions between these two genomes belong to nine different general subsystems including those related to commensalism such as riboflavin metabolism; intracellular resistance; and resistance to antibiotics and toxic compounds (RATC) (Supplementary Table 2). We only found two similarity hits associated with RATC. Complimentary analysis pointed out a bifunctional riboflavin kinase/FMN FMN adenylyltransferase among the best reciprocal similarity's hits between the mycoplasma MAG in this study and *Mycoplasma penetrans* (Fig. S5; Supplementary Table 3).

To understand genome reduction in the mycoplasmas lineage, the genome size and genes count were compared across 247 strains (Fig. 5A; Supplementary Table 4) of the mycoplasmas available in the integrated microbial database (IMG). These strains include a wide variety of human and animal sources and comprising both parasitic and commensal. Given the collected data (Supplementary Table 4), also, this study mycoplasma MAG, gene content and genome size are strongly correlated. The average genome size of the 247 available mycoplasmas was 0.87 Mbp ± 0.15, and the average genes count was 790 ± 157 genes; however, this was not the case with all considered genomes. For instance, 8

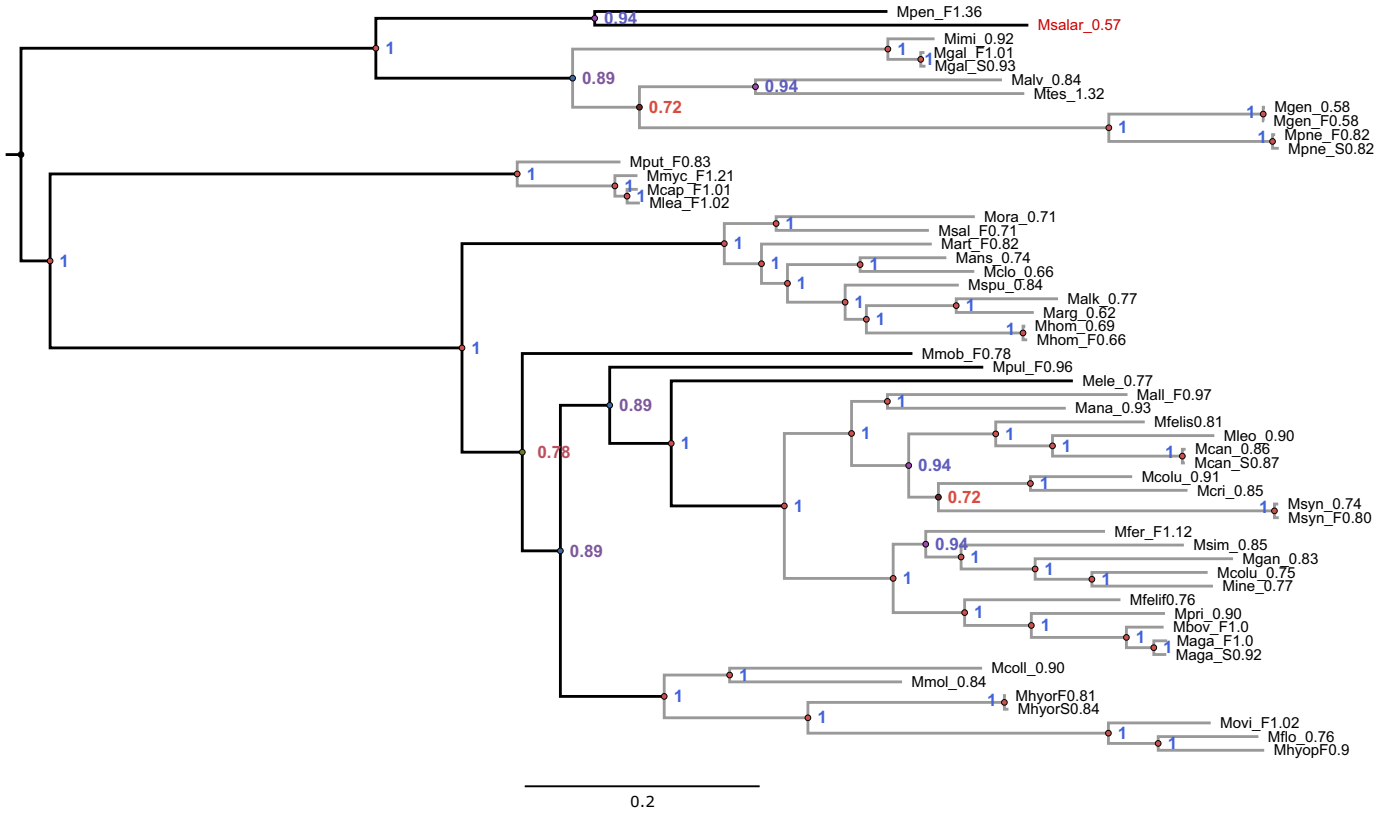

Fig. 3. Phylogenetic tree of mycoplasmas based on 21 MLST markers with the details given in the supplementary data. Sequence name abbreviation of tree tips labels is explained in Supp. File 1 (sheet 3). The tree as consrtucted using MrBayes software , the nodes labels represent the calculated posterior probability values (see Materials and methods).

genomes are lower than 0.8 Mb, accumulating somewhere between 829 and 1036 genes. Further analysis revealed that pseudogenes count had no relationship with genome size whilst both the transmembrane proteins and GC content were correlated with *Mycoplasma* sp. genome sizes (Fig. 5B). Furthermore, the average count of pseudogenes was significantly higher in free-living than within intracellular mycoplasmas (Supplementary Fig. S4), although available databases contain incomplete information with regards to mycoplasmas lifestyles. Finally, enzyme content was analysed in terms of metabolic pathways by comparing the annotated EC numbers of the mycoplasma MAG and *M. penetrans*. Common pathways of both genomes are highlighted in red lines (Fig. 6) including the riboflavin biosynthesis pathway.

4. Discussion

Mycoplasmas are hyper-abundant commensals of salmonid guts. Our study suggests, based on FISH data, that in *Salmo salar*, these organisms grow intracellularly in the epithelial and possibly muscular lining of the fish's GI tract, both in freshwater and during marine lifecycle stages. Mycoplasmas were not visualised on salmon ova, but we cannot rule out vertical transmission between individuals. The *Mycoplasma* sp. sequences recovered from *Salmo salar*, including the mycoplasma MAG reported here, had a strong phylogenetic similarity to *M. penetrans*. Comparative analysis of genome size and content across *Mycoplasma* sp. strains suggest that the genome we recovered in this study is, to the best of our knowledge, among the smallest ever observed. Comparative genomics analyses between the mycoplasma MAG and *M. penetrans* were undertaken and provide insight into the potential host-microbe interaction. Several features of the *Mycoplasma*'s genome organisation and content suggest a strong level of dependence on the salmon host, as well as a potential role for nutrient provisioning relevant to aquaculture.

Mycoplasmas have been widely reported within *Salmo salar* (Holben et al., 2002), and other teleosts (Cheaib et al., 2021; Ei-Jakee, 2020; Sellyei et al., 2021). It is not uncommon to find that communities of gut microorganisms are dominated by mycoplasmas (Dehler et al., 2017). The modelling approaches comparing environmental and intestinal frequency distributions of these organisms undertaken in this study have previously suggested that salmon mycoplasmas are well adapted to the colonisation of their hosts (Heys et al., 2020). Culture-based approaches have had been less successful in isolating these organisms (Llewellyn et al., 2014) and despite numerous attempts, we failed to obtain pure cultures of *Mycoplasma* sp. from the adult salmon used in this study (data not shown). This may be attributed to a potential source of bias arising from cell wall deficiency (Razin, 1995) in mycoplasmas which decreases their growth in presence of inhibitors such as nucleoside and nucleobase as demonstrated in *Mycoplasma pneumoniae* (Sun and Wang, 2013) *and others mycoplasmas* (Wehelie et al., 2004). FISH data from the current study, however, indicate that many mycoplasmas could be sequestered within the basal the epithelial cells, suggesting potential unknown parameters in symbiosis with *Salmo salar* which were missed from the culture media trials and reduce their cultivability. Although only a qualitative assessment is possible by employing FISH, consistent with recent 16S amplicon-seq data from the same farm site (Heys et al., 2020), our data suggest that *Mycoplasma* comprised the majority of the resident microbes (Fig. 2).

A high level of adaptation to, and dependence on the host organism, is a key feature of many *Mycoplasma* species (Faucher et al., 2019). The exploitation of an intracellular niche, dependence on the host, and relative isolation from the other microorganisms and mobile genetic elements are thought to have contributed to genome decay in mycoplasmas (Sirand-Pugnet et al., 2007). One result of this decay is a reduction in genome size and the number of genes, and the mycoplasma

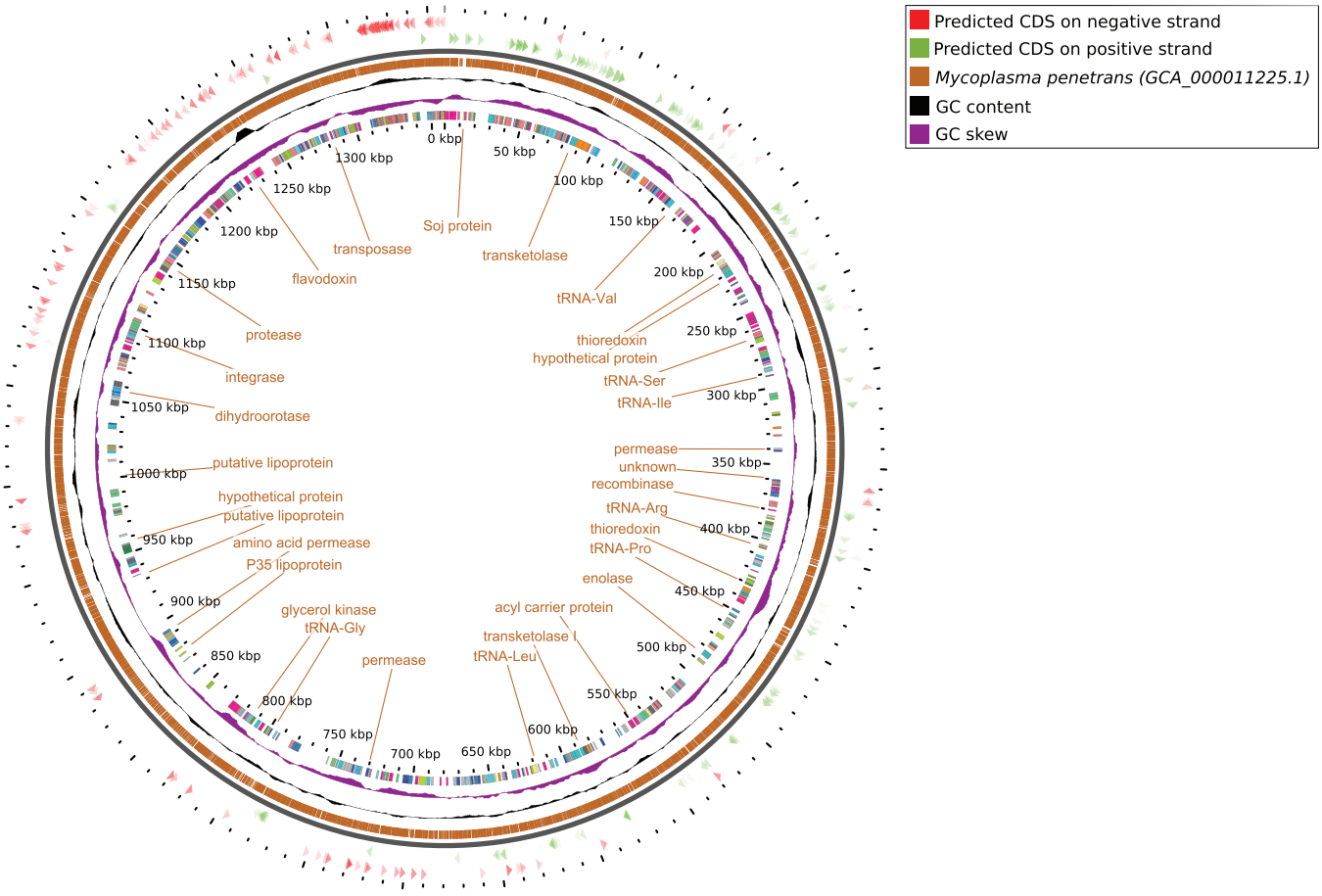

Fig. 4. Circular track of the core genes. This figure highlights the orthologs genes shared between the mycoplasma MAG from this study and related *Mycoplasma penetrans* species.

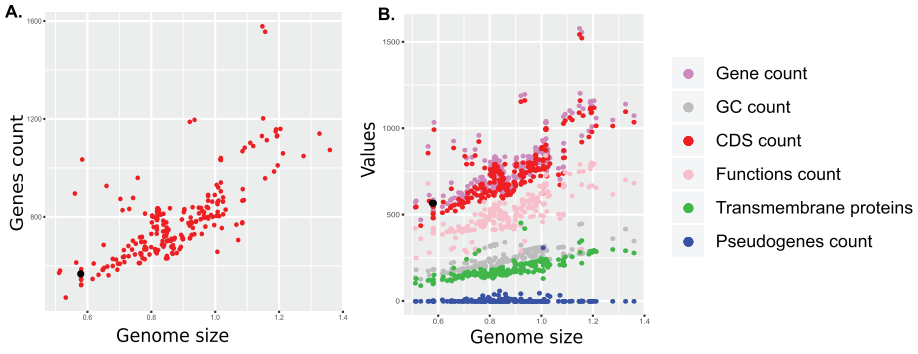

Fig. 5. Genomic features of Mycoplasmas. (A) The plot of genome size and genes count in the Myoplsamas lineage. (B) The plot of functions and GC contents against genome size in the Mycoplasmas lineage.

MAG in this study appears to have been potentially affected by such processes in comparison to the other mycoplasmas (Fig. S5). According to the phylogenetic tree, we did not observe any specific relationship between the tree topology and the genome sizes of mycoplasmas (Fig. 2). Indeed, the closely related *M. penetrans* was over three times larger than the size of the mycoplasma MAG in this study. Despite sharing a recent ancestor with the human pathogen *M. penetrans*, a long and close evolutionary association of this *Mycoplasma* and salmonids is possible given the similarity of another mycoplasma MAG sourced from the Norwegian sea salmon and identified to *M. penetrans* (Jin et al., 2019). We were also able to identify *Mycoplasma* sp. in freshwater parr *via* the FISH method in this study. One potential route for vertical transmission of the *Mycoplasma* sp. among salmon could be observed during oviposition. We were not able to identify microbes colonising eggs in this study, although our sample size was limited. Further development on specific *Mycoplasma* sp. strain markers could potentially reveal their abundance as well as their epidemiology, and potential routes of intergenerational transmission.

Some insight for the potential role of these mycoplasma on salmon health in an aquaculture setting may be possible. Many well-characterised mycoplasmas are pathogens (Meseguer et al., 2003; Rosengarten et al., 2000; Sasaki et al., 2002), with several *Mycoplasma* sp. being responsible for human, animal and plant diseases; however, some species are considered to be commensal organisms (Razin et al.,

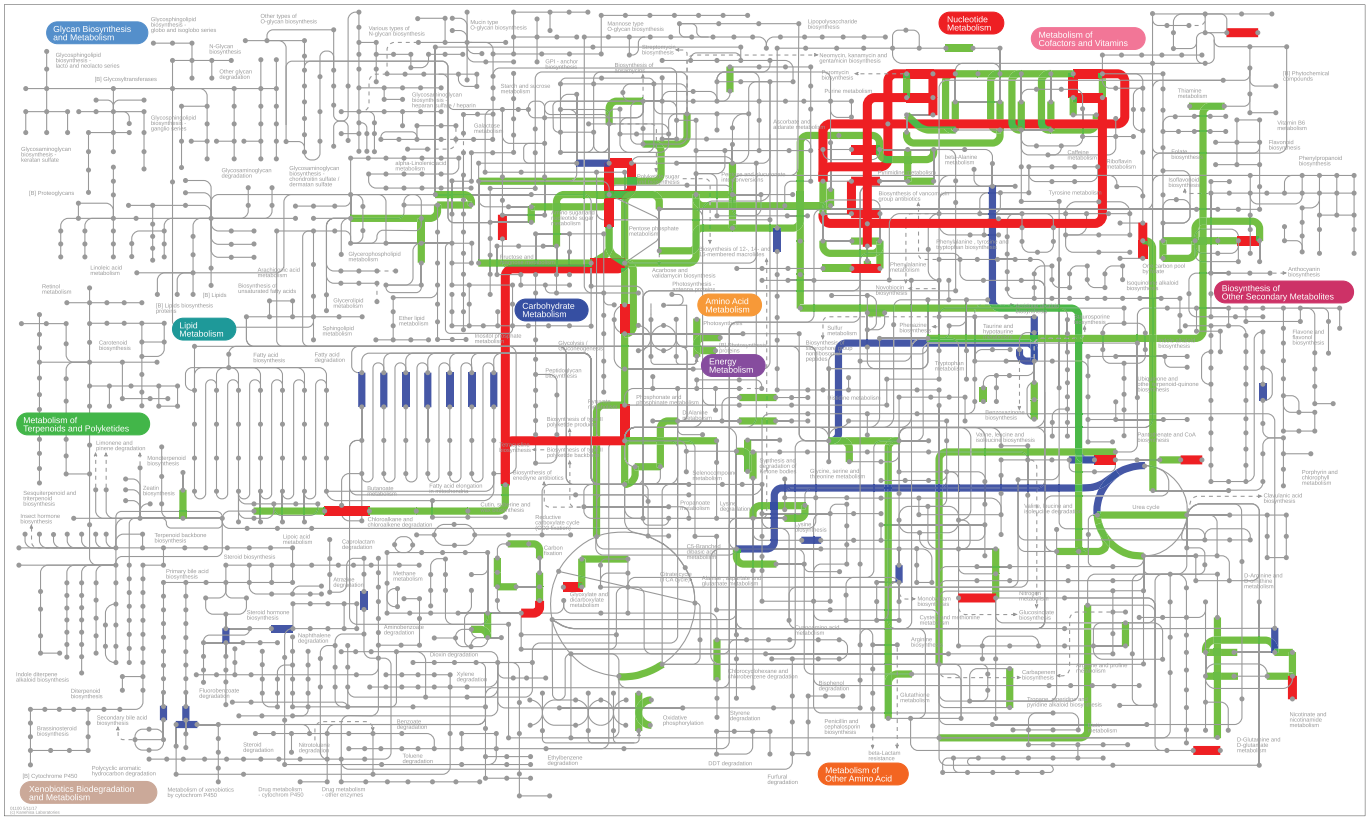

Fig. 6. Pairwise Metabolic pathways comparison of mycoplasma MAG from this study and Mycoplasma penetrans. Red colour represents shared and conserved pathways between the two genomes, whereas blue colour represents the metabolic pathways of the mycoplasma MAG from this study and the green colour represent the metabolic pathways of *Mycoplasma penetrans*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1998; Siqueira et al., 2013). The role of *Mycoplasma* in the context of *Salmo salar* is not well established. Koch's postulates were not applied in this study (Falkow, 2004). Given the challenges encountered in culturing these microorganisms, it seems quite likely that they may never be applied. Furthermore, the apparent abundance of *Mycoplasma* in the healthy salmonids (Heys et al., 2020; Holben et al., 2002; Llewellyn et al., 2016), and lack of any clear associated pathology in gut tissues, implies that there is not a significant impact on the host health or fitness. Commensal exploitation of the host intracellular niche is potentially the most parsimonious description of the host-microbe interaction in this case. The ultimate metabolic adaptation to an intracellular lifestyle (i.e *Buchnera*, *Wigglesworthia* and *Blochmannia)* appears to be solely regulated by the metabolic activity of the host cells to which the bacteria may actively contribute to, by delivering essential metabolites that are limited in their habitats and are not produced by the hosts (Fuchs et al., 2012). In this context, the presence of genes encoding riboflavin pathway could potentially indicate benefit from the salmon host perspective of *Mycoplasma* sp. colonisation. Riboflavin, known as the precursor for the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide, is an essential metabolite in organisms (Fuchs et al., 2012; Gutiérrez-Preciado et al., 2015), although vertebrates cannot synthesize it on their own (Vitreschak et al., 2002). The *Mycoplasma* may play a role in riboflavin supplementation in salmon, as has been suggested in several deep-sea snailfish (Lian et al., 2020), although it must be noted that riboflavin biosynthetic pathways also occur in pathogenic mycoplasmas (Gutiérrez-Preciado et al., 2015). Riboflavin supplementation is not limited to the mycoplasmas; in the bedbug *Cimex lectularius,* the gram-negative Wolbachia can synthesize biotin and riboflavin which, are crucial for the host growth and reproduction (Kubiak et al., 2018; Moriyama et al., 2015). Riboflavin biosynthesis is common for symbiotic associations and therefore occurs even in small and optimized genomes size like *Wolbachia* (~ 1.48 Mb) *and mycoplasmas* (0.51–1,38 Mb).

Also, it is reported that many *Mycoplasma* species can modify their surface antigenic molecules with high frequency (Horino et al., 2003; Rosengarten et al., 2000) which may likely play a key role in outmanoeuvring the host immune system. This ability may generate phenotypic heterogeneity in colonising Mycoplasma populations and provide fitness benefits such as evasion of host immune responses and to the adaptation to the environmental changes (Halbedel et al., 2007; Horino et al., 2003). The majority of the variable surface antigenic molecules of mycoplasmas are lipoproteins (Chambaud et al., 1999; Halbedel et al., 2007; Wise, 1993), which, depending on the species, are encoded by single or multiple genes (Rosengarten et al., 2000; Rosengarten and Wise, 1990). The expression of these lipoproteins, due to extensive antigenic variation, is thought to be a major factor for immune evasion, for example, the P35 lipoprotein and its paralogs, which are distributed across the surface of *M. penetrans* cells, are immunodominant (Distelhorst et al., 2017; Neyrolles et al., 1999; Wang et al., 1992). Two lipoprotein encoding genes were found only in *Mycoplasma penetrans* but not in the mycoplasma MAG of this study, and the lack of such virulence factors or mobile genes could again support a non-pathogenic lifestyle (Supplementary Table 2).

Our work demonstrates a potentially important ecological and functional association between *Mycoplasma* sp. and *Salmo salar* that merits further investigation in the context of aquaculture disease and, potentially, nutrition. Targeted meta-transcriptomics and strain-specific screening for this organism could improve our understanding of its biology, function, and its role in the host homeostasis. Furthermore, targeted studies involving genome reduction and their association with

the host dynamics are also necessary to fully understand the evolution of *Mycoplasma* sp. symbiosis in *Salmo salar*. Furthermore, bespoke infections experiments, informed by the findings of this study, may lead to the development of practices that can improve the aquaculture industry, especially in the context of the probiotic potential of mycoplasmas in salmonids.

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ML and BC conceived and designed the study. PY, RK, MDN, TD, CH, PS, and EL collected the samples, performed FISH experiments, and analysed image data. BC performed the bioinformatics and interpreted the results. BC and ML wrote the original draft of the manuscript. All authors contributed to revisions of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736772.

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