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# Mycoplasma pathogenicity for humans and animals: an effort of complete genome sequencing and gene family analysis of *Mycoplasma synoviae*

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Like humans, chickens can become sick from bacterial infections. Mycoplasma is a very small, slow-growing bacterium. There are multiple types of Mycoplasma that affect different animals, humans, and even plants and insects. In the current study, the whole genome of *Mycoplasma synoviae* was sequenced through the joint sequencing method of the third-generation PacBio and second-generation Illumina of the high-throughput sequencing platform, and the complete genome map of the Mycoplasma synoviae was successfully obtained. SMRT sequencing and assembly of whole genome: Canu v1.5/ WTDBG v2.2 software was used for preliminary assembly of filtered subreads. Pilon V1.22 software was used to further correct the assembled genome with high-quality second-generation data, and the genome with higher accuracy was finally obtained. Sequencing results showed that the Mycoplasma was composed of a 0.83Mb chromosome with a G+C percentage of 28.42%. Bioinformatics was used to predict the encoding protein genes, and it was found that Mycoplasma synoviae genome encoded a total of 1415 ORF open reading frames, including 1.73% of the repeats, 7 ribosomal RNAs, 34 transport RNAs, 4 non-coding RNAs and 12 pseudogenes. Mycoplasma synoviae genomes also contain 2 CRISPR, 1 gene island and 2 prophages. Using multiple strains of Mycoplasma gallisepticum, Mycoplasma iowae and Mycoplasma anatis as reference genomes, we constructed phylogenetic trees of 12 species of Mycoplasma. The genetic and taxonomic status of Mycoplasma synoviae strains were evaluated. The results showed that the sequencing strains of Mycoplasma synoviae and the model strain Mycoplasma synoviae WVU1853 were closely clustered together, suggesting the genetic relationship was relatively close and the sequencing strains were relatively conservative, and represent that they share the same genetic origin.
Keyword: Mycoplasma synoviae, genetic analysis, Sequencing, DNA, RNA.

## INTRODUCTION

The discovery of double helix structure model of DNA molecule has encouraged the life science researcher four further discoveries on molecular level (Johansson and Pettersson, 2002). In 1975, Sanger and his co-worker made millions of identical copies of a DNA template by cloning or polymerase chain reaction (PCR), and completed the genome sequencing of bacteriophage X174, with a total length of 5375 bases, that has successfully opened the era of first-generation sequencing.

First generation sequencing usually refers to the chain termination method or chain degradation method, whose core is deoxyribonucleotide triphosphate (dNTPs) which interrupts the DNA synthesis reaction. The first human genome sketch was completed by using the first-generation sequencing technology (Feberwee *et al.*, 2009). The generation of first-generation sequencing has greatly promoted the research of modern molecular biology (Kang *et al.*, 2002). The sequencing read length of first-generation sequencing (Sanger sequencing) reached at 1000bp, but its low throughput and high cost, Sanger sequencing is used for single sequence determination (Morrow *et al.*, 1998). To overcome these deficiencies, researchers carried out working on sequencing technology, and now the second-generation sequencing technology has emerged at the historic moment. In the second-generation sequencing technology, the DNA samples to be tested is divided into small sequence fragments of 200-500bp by ultrasonic wave, and the DNA is amplified by bridging PCR on the surface of the sequencing instrument

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using the coupling method (Jones *et al.*, 2006). Finally, the optical signal is transformed into the sequencing base recognition by using the method of synthesis while sequencing, and the determination of specific sequences is completed (Shahid *et al.*, 2013). Second-generation sequencing technologies include Roche's 454 technology, Illumina's Solexa, Hiseq technology and ABI's Solid technology, however, second-generation sequencing technology is still the mainstream technology in the field of life science. Second-generation sequencing quickly completes the determination of gene sequences, and transforming scientific research from a single local gene to gene fragment study of the entire genome, which is widely being used in the de novo sequencing and transcriptome sequencing of genomes (Zhu *et al.*, 2018). Second generation sequencing technologies produce a massively parallel analysis with a high throughput and can sequence millions to billions of reads in a single run and the time required to generate the GigaBase sized reads is only a few days or hours making it best than the first-generation sequencing such as Sanger sequencing (Noormohammadi *et al.*, 2003).

Yet the second-generation sequencing technology had several flaws limiting the research on DNA single base molecules, that are overcome by sequencing third-generation sequencing technology (Lockaby *et al.*, 1998). Whole genome sequencing is the method to obtain the genome map of a species through *de-novo* sequencing of non-parametric species, stitching's and assembly by means of bioinformatics. High-quality reference genome is the basis for modern molecular biology to deeply understand the genetic composition and evolutionary traceback of species (Green and Sambrook, 2016; Green and Sambrook, 2017). *Mycoplasma* is often used as a model for the study of minimal life, which has the smallest genome among known prokaryotes. In the late 20th century, researchers completed the entire genome assembly of *Mycoplasma genitalium* G37 by shotgun sequencing, which was the first *Mycoplasma* to obtain a reference genome (Andrews, 2010). Papazisi*et al.* (2003), in 2003 performed whole genome sequencing on R strain of *Mycoplasma gallisepticum* (AE015450). Vasconcelos *et al.* (2005), in 2005, completed the assembly of *Mycoplasma synoviae* of Brazil 53 strains (AE017245) (Zerbino and Birney, 2008). Second-generation and third-generation sequencing technology have brought the revolution in the genomics of *Mycoplasma* rapidly. 'By using these technologies, *Mycoplasma synoviae*, *Mycoplasma gallisepticum* and *Mycoplasma iowae* strains of poultry *Mycoplasma* had completed genome sequencing assembly'. To date, by using these technologies, *Mycoplasma* strain has reached more than 50 strains. However, further research is required to explore the further strain of *Mycoplasma*. Therefore, current study was conducted to analyses complete genome sequencing of *Mycoplasma synoviae*.

## MATERIALS AND METHODS

***Feeding and management:*** Birds were fed on the standard feed as described in the literature (Hussain *et al.*, 2018; Xu *et al.*, 2019; Arshad *et al.*, 2020; Bajwa *et al.*, 2020; Hussain *et al.*, 2020; Kamran *et al.*, 2020; Shahid *et al.*, 2020; Sharif *et al.*, 2020; Shahid *et al.*, 2021). Birds were managed according to standard procedure as described in literature (Hussain et al., 2018; Xu et al., 2019). Birds were free from hunger and thirst and manged according to standard protocol of animal behavior and welfare (Muhammad *et al.*, 2016; Aziz ur Rahman *et al.*, 2017; Aziz ur Rahman *et al.*, 2019; Muhmmad *et al.*, 2020).

***The source of Mycoplasma synoviae:*** The Ningxia University laboratory had already identified and preserved the Ningxia G-1 strain

***Media and culture conditions:*** *Mycoplasma synoviae* special medium

***Main instrument of the experiment and reagents:*** Instrument and reagents used in the current experiment were Ultra clean table, Supplement instrument manufacturer, Biochemical incubator, Micro tissue grinding machine, Ultra-micro UV spectrophotometer (Li *et al.*, 2014; Niu *et al.*, 2017; He *et al.*, 2018; Xia *et al.*, 2018a; Xia *et al.*, 2018b; Li *et al.*, 2019; Qiu *et al.*, 2019a; Qiu *et al.*, 2019b; Qiu *et al.*, 2020), Fluorescence real-time quantitative PCR instrument (Qiu *et al.*, 2018; Rehman *et al.*, 2019; Chen *et al.*, 2020), Electrophoresis instrument, Sequencing instrument (Li *et al.*, 2019; Qiu *et al.*, 2019a; Qiu *et al.*, 2020), Pacific Biosciences. Genomic DNA Extraction Kit; Accel-NGS®·XL; Qubit™

***Experimental method***: *Culture of strain and whole genome sequence*

According to *Mycoplasma synoviae* strain amplification culture conditions, liquid culture, authenticate, DNA gene extraction by *Mycoplasma synoviae* was used as a template. Complete genome sequencing and assembly of *Mycoplasma synoviae* were performed based on PacBio third-generation sequencing platform. The main processes include

***Extraction and purification of whole genome DNA and genomic DNA fragmentation:*** In this study, the Genomic DNA of *Mycoplasma synoviae* was extracted using Qiagen genomic-tip kit, and the whole process was carried out according to Protocol procedures. The Accel-NGS XL kit was used to prepare the genomic DNA library with the inserted fragment length of about 20Kb, which mainly included DNA fragmentation, fragment repair, addition of adapters, purification of DNA and fragment selection.

***Construction of third-generation sequencing genomic DNA library:*** 10-15μg of high-quality whole genome DNA-SMRT Bell library was sent to Beijing Biometrics for sequencing. The sequencing platform relied on the third-generation single-molecule real-time sequencing machine, PacBio, and the library size was 20Kb.Primers for full sequence of vlhA gene are presented in table 1. Raw data from the sequencing

machine included DNA adapters and low-quality data. Quality control of sequencing data was completed by further filtering adapters and short reads to obtain total data sets (subreads). The number of subreads, the total number of bases of subreads, the value of subreadSN50, the average number of bases and the distribution of reads length were counted respectively to ensure the authenticity and reliability of the sequencing results. The details of PCR reaction system and conditions are presented in table 2 and 3, respectively.

**Table 1. Primers for full sequence of vlhA gene.**

| NAME | Primer | Sequence | Length |
|---|---|---|---|
| vlhA | vlhA-f | 5'-GCCATTGCTCCTGCTGTTATA-3' | 773bp |
| | vlhA-r | 5'–GGGTAGTCCACTCGCATT-3' | |

**Table 2. PCR reaction system**

| Reagent | Volume |
|---|---|
| Dd $H_2O$ | Up to 50 ul |
| 2×Phanta Max Master Mix | 25 ul |
| forward primer | 2 ul |
| reverse primer | 2 ul |
| vlhA Form | 2 ul |
| total volume | 50 ul |

**Table 3. PCR reaction conditions.**

| Step | Temperature | Time | circulation |
|---|---|---|---|
| predegeneration | 95 ℃ | 4 min | |
| denaturation | 95 ℃ | 40 sec | 30 |
| anneal | 56 ℃ | 40 sec | |
| extend | 72 ℃ | 60 sec | |
| Completely stretch | 72 ℃ | 10 min | |
| save | 04 ℃ | | |

The assembled contig sequence was compared with the NT database, and finally assembled into a circular genome, that is, to complete the 0GAP genome completion map sequence. The software Circos was used to draw the genomic circle map, showing the relationship between genome components or locations more clearly.

***Whole genome analysis***

***Genomic component analysis:*** Repeated sequences in Mycoplasma genome were predicted by REPEATMASKER V4.0.5 software. Prodiga V2.50 software was used to predict the coding genes of the assembled genome. The software trnascan-se v1.3.1 was used to predict the tRNA in the genome, and the software Infernal V1.1 was used to predict the rRNA in the genome and other ncRNAs besides tRNA and rRNA based on the RFAM v12.0 database. Bioinformatics analysis software Genblasta v1.0.1 was used to predict the protein sequence and carry out sequence alignment with the proteins of the same family in the Swiss-Prot public database, so as to retrieve the gene sequences homologous with the predicted genes on the genome. Bioinformatics software Genewise Wise2-2-0 was further used to mine the immature stop codon and frameshift mutation in the gene sequence to obtain pseudogenes.

***Functional annotation of genome:*** GO database was used for gene functions and analysis.

## RESULTS AND DISCUSSION

Results of genomic DNA extraction are presented in Fig. 1. The general features of the *Mycoplasma synoviae* genomes are listed in Table 4.

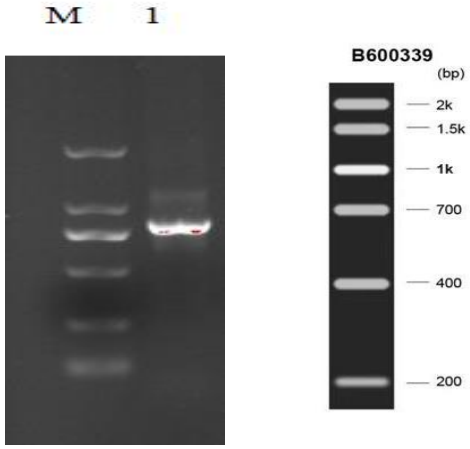


**Figure 1. PCR amplification (M: Marker, 1:vlhA)**

A total of 1.4 GB of filtered high-quality sequencing data were obtained by smart-seq technology, including 153,137 subreads, and the mean subreads length after filtering was 9,109bp. The length of N50 (SubreadSN50) was 11,656bp. After assembling the filtered subreads with CANU software, a contig with a size of 826,450bp was obtained, which completely covered the *Mycoplasma synoviae* genome. The GC Content of *Mycoplasma synoviae* genome was 28.42%. Through multiple annotation and prediction, *Mycoplasma synoviae* genome contains a total of 1,415 protein coding genes, the total predicted gene length was532,710bp, accounting for 64.46% of the whole genome,7 ribosomal RNA (rRNA), 34 transport RNA (tRNA), Four other non-coding RNAs (ncRNAs) and 12 pseudogene genes were identified.

Gene islands can be related to a variety of biological functions and can be divided into different subclasses based on their different functions. *Mycoplasma synoviae* genome contains a gene island with a length of 3,402bp and a total of 10 protein-coding genes. The presence of Prophage sequences may enhance the host's adaptability to the environment. The predicted results showed that *Mycoplasma synoviae* genome contained two prophages, with the length of 39,667bp and 11,430bp, respectively. As expected, the *Mycoplasma synoviae* mutation in the obg gene (Shahid *et al.*, 2013), previously reported as a marker of the vaccine strain, was identified.

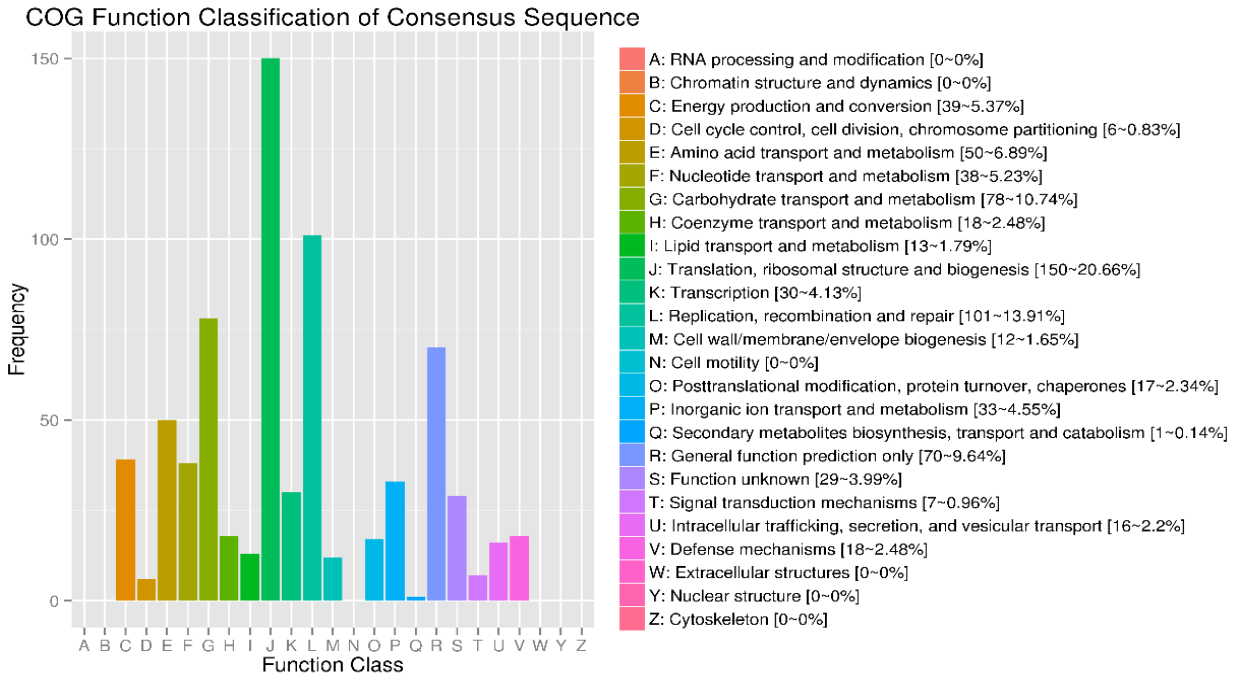


**Figure 2. COG database function notes**

**Table 4. Genome statistics.**

| Attribute | Number | Full/mean length (bp) |
|---|---|---|
| Genome | 1 | 826,450 |
| Total genes | 1,415 | 532,710 |
| RNA genes | 45 | - |
| PesudoGene | 12 | 6,764 |
| CRISPR | 2 | 36 |
| Gene island | 1 | 3,402 |
| Prophage | 2 | 25,549 |

COG is a database containing the Orthologous gene clusters of prokaryotes. Proteins that form each Orthologous group are assumed to be from an ancestor protein with similar functions. The potential function of prokaryotic genes can be annotated by BLAST comparison of the database. The results of COG annotation functional classification of *Mycoplasma synoviae* genomic data are shown in Fig. 2.

A total of 696 coding genes were annotated into 19 COG functional clusters.150 genes were annotated into translation, ribosomal structure and biogenesis functional protein clusters, accounting for 20.66% of the total annotated genes. The basic breakdown of proteins into functional categories is similar to other mycoplasmas, with an emphasis on transport of compounds and protein synthesis (Westberg *et al.*, 2004). As is typical of a newly sequenced genome, one-fourth to one-third of all proteins are without a well-annotated function. For genes that could be placed in clusters of COGs, the functional category was automatically determined. For genes that did not match to COGs, the gene was assigned to a COG category manually, with the majority of such cases being assigned to the "Function Unknown" category. For genes with more than one COG category, only the first one (in alphabetical order) was used. Amino acid transport and metabolism, energy production and conversion, and energy production of carbohydrates and conversion), nucleotide transport and metabolism, replication, (recombination and repair) and general function prediction only, the number of genes identified by functional annotations exceeded 5%. Only one gene was involved in secondary metabolites biosynthesis, transport and catabolism.

These results showed that the functions of most genes were still focused on supporting the growth and development of living organisms and energy metabolism, and the results of classification annotation were basically consistent with the commonness of mycoplasma. At the same time, 29 genes were annotated as functionally known genes by COG database, accounting for 3.99%, and their functional potential is yet to be explored. The genome encodes numerous transporters with a wide range of substrates and possesses some additional transporters of unknown specificity. It appears that *Mycoplasma synoviae* should be able to transport and metabolize glucose, sucrose, fructose, maltose/maltodextrin, xylose, and trehalose as energy sources. This has been shown experimentally for glucose and sucrose (Jaffe *et al.*, 2004). Glycerol should also be able to be used as an energy source, but no specific transporter for glycerol was found. The presence of mannose-6-phosphate isomerase (*manA*) suggests that mannose may also be metabolized, as is suggested elsewhere (Pollack, 2002). As is common to other mycoplasmas, *Mycoplasma synoviae* should

be able to produce and use glycogen and starches. Fermentation of sugars appears to be the only method of ATP production in *Mycoplasma synoviae*. A complete glycolysis pathway is present that terminates in the formation of lactate. Most of the nonoxidative branch of the pentose phosphate pathway is present except for the reaction normally carried out by transaldolase. This is similar to the situation in *Mycoplasma synoviae* and other mycoplasmas, and presumably this function is carried out by an as-yet-unrecognized protein (Pollack, 2002; Westberg *et al.*, 2004). A variety of DNA restriction/modification system enzymes are detected in *Mycoplasma synoviae*. This may account for their recalcitrance to genetic manipulation via transposon mutagenesis (J. Jaffe, unpubl.; M. Miyata, pers. comm.) even though it is a popular mutagenesis technique in other mycoplasma species. A similar obstacle was overcome in *Mycoplasma arthritidis* by using an appropriate DNA modification enzyme to circumvent that species' endogenous restriction enzymes (Voelker and Dybvig, 1996). GO(Gene Ontology) database contains the annotation of target genes and their products, which is based on cellular component, molecular function and biological process.

The aim is to define and characterize the function of genes and proteins in multiple species. As shown in Figure 3, a total of 912 genes in *Mycoplasma synoviae* genome were annotated into GO database, among which 392 genes in cell components were annotated into cell-related terms. In the molecular function module, the number of genes related to catalytic activity and binding was the highest, 658 and 581, respectively. Annotation genes were mainly found in metabolic process and cellular process, which contained 595 and 534 genes respectively (Fig. 3).

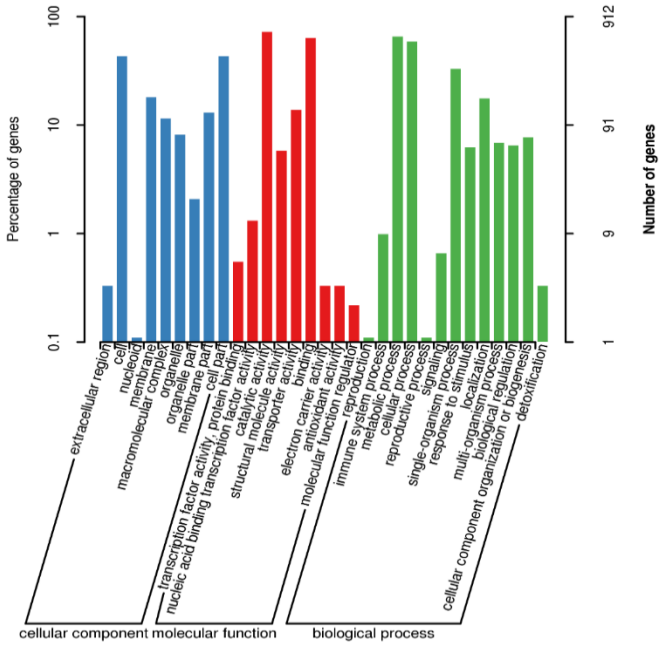


**Figure 3. Go database function notes**

The results showed that some items of biological process were involved in regulating cellular process, single-organism process and metabolic process; some items of cellular component were involved in regulating cell part, intracellular membrane-bounded organelle; and some items of molecular functions were involved in regulating protein binding, organic cyclic compound binding and catalytic activity as reported elsewhere (Zhao *et al.*, 2020).

***Conclusion*:** Based on results it is concluded that the sequencing strains of *Mycoplasma synoviae* and the model strain *Mycoplasma synoviae* WVU1853 were closely clustered together, suggesting the genetic relationship was relatively close and the sequencing strains were relatively conservative, and represent that they share the same genetic origin.

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