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A core genome multi-locus sequence typing scheme for *Mycoplasma hyosynoviae*

Diploma thesis

University of Veterinary Medicine Vienna

submitted by

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TABLE OF CONTENTS

Dedication

For Ernestine.

Abbreviations

AFLP	amplified fragment length polymorphism
Bp	base pairs
cg	core genome
DNA	deoxyribonucleic acid
e.g.	*exempli gratia*
i.e.	*id est*
kb	kilo bases
M.	*Mycoplasma*
MALDI-TOF	matrix assisted laser desorption/ionization - time of flight
Mb	mega bases
min	minutes
MLST	multi locus sequence typing
MLVA	multi locus variable number of tandem repeats analysis
MS	mass spectrometry
MST	minimum spanning tree
NCBI	National Center for Biotechnology Information
NJT	neighbor joining tree
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
RAPD	randomly amplified polymorphic DNA
STs	sequence types
rpm	rounds per minute
USA	United States of America
vapD	virulence associated protein D
VNTR	variable number of tandem repeats

WGS	whole genome sequences

1. Introduction

Mycoplasma (*M.*) *hyosynoviae* is a host specific pathogen of the class *Mollicutes,* which is adapted to pigs. This parasitically living microorganism causes mainly non purulent arthritis in grower or finisher pigs. This causes on the one hand economic losses through a worse feed conversation ratio and increased mortality, and on the other hand animal welfare issues through severe pain while moving. Data on the prevalence of the pathogen in the field are lacking, but there are case reports of *M. hyosynoviae* infections all around the world. Therefore, it is believed that this pathogen is endemic in most big scale pig production units. It is assumed that in a naive herd the microorganism spreads gradually throughout the whole farm, emanating from few persistently infected carrier animals. It is noteworthy that not all colonized pigs exhibit signs of an infection (i.e., clinical apparent arthritis). Clinical outbreaks can usually be stemmed *via* the administration of antibiotics, through which abatement of the symptoms may be achieved within days. However, decreased susceptibility of *M. hyosynoviae* to some classes of antimicrobials were reported in the past. Commercial vaccines are currently not available. Results of experimental autogenous vaccination trials are unfortunately sobering.

Microbiological examination of sampling material can often result in dissatisfactory outcomes despite clear clinical and pathoanatomical findings for potential pathogen involvement, due to the challenging and tedious cultivation requirements of *M. hyosynoviae.* Therefore, in the field PCR is the most commonly used method for routine diagnostic purposes. The advantage of these techniques is that there is no need for pathogen cultivation and non-viable organisms can be detected as well. For epidemiological investigation of *M. hyosynoviae* infection molecular typing techniques providing sufficient discriminatory power are required. Recently, core genome multi locus sequence typing (cgMLST) schemes were proposed to be applied for such investigations on other mycoplasmas. For example, schemes for *M. gallisepticum, M. synoviae* and *M. anserisalpingitidis* were recently established and successfully employed for epidemiological investigations and outbreak analyses. Moreover, a cgMLST scheme for *M. hyorhinis* is currently in development.

The aim of the presented diploma thesis was the development of a cgMLST scheme for the accurate typing of *M. hyosynoviae* isolates. To achieve this goal, genomic data of 51 *M. hyosynoviae* strains originating from Austria and Germany and nine publicly available genomes from strains isolated in Denmark, Canada and the USA were included in the study.

2. Literature review

Etiology and epidemiology of *Mycoplasma hyosynoviae*

Mycoplasma hyosynoviae was first described as a separate porcine mycoplasma species by Ross and Karmon (1970). Taxonomically it belongs to the class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae*, genus *Mycoplasma* and therein it is phylogenetically placed in the Hominis cluster of the Hominis group, according to 16S rRNA gene sequences (Peters et al. 2008, Siqueira et al. 2013, Volokhov et al. 2012, Weisburg et al. 1989). The pathogen's natural host is the swine where it is primarily localized in the *tonsilla palatina*. This microorganism may persist in the tonsils throughout the host's lifespan (Kobisch and Friis 1996).

Like other *Mollicutes*, *M. hyosynoviae* lacks a cell wall and is small in size. Due to the devoid of fermentative capability, this microorganism relies on the arginine dehydrolase pathway to produce energy. It does not possess the ability to hydrolyze urea. *M. hyosynoviae* is rapidly propagating in and on specialized media for the cultivation of mycoplasmas. Modified Hayflick's medium supplemented with mucinous material from swine is best suited for the cultivation of *M. hyosynoviae* (Kobisch and Friis 1996). Colonies of *M. hyosynoviae* develop within 2 – 5 days on agar plates, exhibit fried egg morphology and are rather small (0.1 – 0.5 mm in diameter). Ammonia is released from arginine hydrolysis, which results in an alkaline pH and a color change of the medium to red.

Up to date, one complete genome of a *M. hyosynoviae* strain (CP008748, strain M60 cultivated from a sample of an arthritic swine joint in 1968) and draft genomes of eight additional strains including the type strain (ATCC 25591) have been published and/or deposited at the NCBI genome database (National Center for Biotechnology Information 2021). The completed genome of strain M60 consists of a circular chromosome of 864 kilo bases (kb), with an overall guanin and cytosin content of 27%. Detailed comparative genomic analyses have only been reported on seven *M. hyosynoviae* draft genomes (Bumgardner et al. 2014, Bumgardner et al. 2015) resulting in the identification of genes associated with antibiotic resistance and of putative virulence factors. Like other representatives of the class *Mollicutes, M. hyosynoviae* possesses a gene homolog that expresses the virulence associated protein D (*vapD*) which is commonly found in many other pathogenic bacteria. Various roles have been attributed to *vapD* including roles as an endoribonuclease and a toxin (Kwon et al. 2012). *M. hyosynoviae* also encodes a protein that exhibits structural resemblance to the MAA1 prophage protein

found in *M. arthritidis.* In this rodent mycoplasma species MAA1 has shown to meditate adhesion to synovial membranes (Washburn et al. 2000). Like other *Mycoplasma* species, *M. hyosynoviae* lacks most of the enzymes necessary to perform vital reactions. For example, the microorganism cannot degrade sugars or other substances on its own. It cannot produce amino acids either. *M. hyosynoviae* largely depends on the host's supply of these essential nutrients. Therefore, this pathogen encodes several genes dedicated for nutrient acquirement from the host environment. In particular, *M. hyosynoviae* encodes an *opp* operon containing genes *oppA, oppB, oppC, oppD* and *oppF,* which has shown to transfer ATP and oligopeptides across the cell membrane (Henrich et al. 1999, Oakley et al. 1999). In *M. hominis*, *oppA* has also been described as important virulence factor, as it functions as a cellular adhesin (Oakley et al. 1999). In addition, *oppA* has been shown to function as the primary ecto-ATPase on the surface of the cell and may induce apoptosis of host cells (Hopfe and Henrich 2008, Oakley et al. 1999). Two recent studies have likewise suggested that a complete functioning *opp* operon is necessary in *M. gallisepticum* for full expression of virulence (Masukagami et al. 2018, Tseng et al. 2017).

Information regarding the epidemiology of *M. hyosynoviae* is very limited. It appears that this microorganism is a pathogen occurring worldwide. Furthermore, it is assumed that most of the pig herds in countries with industrialized scale production units are at least colonized with this microorganism (Kobisch and Friis 1996). Case reports from various countries all over the world strengthen this presumption (Kobisch and Friis 1996). Two studies showed that *M. hyosynoviae* cannot be detected via real time PCR in one to seven days old piglets, despite 55 – 60% of their dams being tested positive at the time of farrowing. However, three weeks after farrowing 0,9 – 13% of piglets were tested positive (Roos et al. 2019, Schwartz et al. 2014). Microbiological examinations of samples originating from ten pig herds with a history of acute lameness in finisher pigs between three and five months of age in the Netherlands showed that *M. hyosynoviae* most likely played a significant role in the disease process of those animals (Geudeke et al. 2016). *M. hyosynoviae* has frequently been detected via real time PCR in tonsillar swabs (Gomes Neto et al. 2015, Roos et al. 2019), albeit the pathogen was not detectable in nasal discharges (Gomes Neto et al. 2015). It was speculated that *M. hyosynoviae* either has a differential tropism for tonsils compared to the nasal cavity, or an ecological barrier exists that determines the inhabitance and ecology of these structures or niches (Gomes Neto et al. 2015). Furthermore, *M. hyosynoviae* was reported to be regularly detectable in pen-based oral fluids (Gomes Neto et al. 2015, Pillman et al. 2019) and the

presence of *M. hyosynoviae* in pen based oral fluids correlated significantly with an increased lameness score of tested pens. In addition, an increased detection rate of *M. hyosynoviae* in finisher compared to nursery pigs was observed (Pillman et al. 2019). Multiple factors may cause a delayed detection of *M. hyosynoviae*, such as protective circulating maternal antibodies (Lauritsen et al. 2017), few infected animals at the time of weaning, low rate of transmission, or unknown host factors that confer temporary immunity (Gomes Neto et al. 2015).

Mycoplasma hyosynoviae – pathogenesis and clinical signs

It is commonly assumed that *M. hyosynoviae* is introduced to non-infected herds *via* contact to persistently colonized pigs. Transmission of this microorganism happens trough close animal contact by nasal secretions. It is also believed that the pathogen can be transferred *via* infectious aerosols (Ross and Spear 1973). However, the microorganism is only inconsistently shed *via* nasal secretions (Ross and Spear 1973). During systemic spread *M. hyosynoviae* can be rarely detected in serum or blood prior to the onset of clinical signs (Gomes Neto et al. 2016). The primary site of colonization are the tonsils (Gomes Neto et al. 2016, Kobisch and Friis 1996), and pigs can become persistent carriers (Hagedorn-Olsen et al. 1999). Nielsen et al. (2001) reported that in flocks with high incidence of severe, acute lameness among grower-finishing pigs, the prevalence of tonsil carriers with *M. hyosynoviae* was approximately 75%, but the microorganism was also detected in clinically unaffected animals. In an experimental infection study using specific pathogen free pigs *M. hyosynoviae* was shown to lack an apparent tropism for a specific joint. Tissue invasion and histopathological alterations were detected in multiple joints regardless of the strain inoculated, infection dosage and route of infection (Gomes Neto et al. 2016). Passively transferred colostral antibodies seemed to have only a partial protective effect against the development of *M. hyosynoviae* related pathologies in piglets 4.5 weeks post farrowing (Lauritsen et al. 2017). After pigs were experimentally infected with different *M. hyosynoviae* strains, a serological immunoglobulin G response was evident 17 days post exposure (Macedo et al. 2019). Systemic infections with *M. hyosynoviae* may induce inflammatory and humoral responses, but their protective role remains to be elucidated (Macedo et al. 2019).

M. hyosynoviae induced lameness is most seen in three-to-five-month-old pigs. One or several limbs can be affected, but most animals show symptoms like soft fluctuating joint swellings in the hind legs. Lameness in the front limbs might be masked due to pain in the rear legs and

the spine. In addition, pain while moving, arched backs, stiffness, weight shifting and difficulties getting up are commonly observed. Lame pigs are often reluctant to walk or to bear weight on the affected limbs (Kobisch and Friis 1996, Nielsen et al. 2001). Recent experimental studies have demonstrated that clinical signs emerge one to 15 days post infection, depending on the route of inoculation (Gomes Neto et al. 2016, Macedo et al. 2019). In another study it was shown that *M. hyosynoviae* can be present for weeks in unaffected joints indicating a clinically silent phase in which the microorganism colonizes joints without causing apparent dysfunctions (Nielsen et al. 2001). These findings have also been described by (Kobisch and Friis 1996). Furthermore, *M. hyosynoviae* associated arthritis may often follow stress, e.g., movement of animals to new production units or farms, and lameness may be observed one to three weeks after such movements.

Diagnosis, therapy, and prophylaxis of *Mycoplasma hyosynoviae* infections

Due to the fastidiousness of *M. hyosynoviae* it is of utmost importance to use the correct sampling method and select the appropriate animals for testing. Acutely affected swine without prior antimicrobial treatment are preferred over chronically lame pigs, in which secondary infections may have occurred. Deceased pigs should be avoided for testing due to potential chronicity and lack of proper ante mortem examination. For correct diagnosis of *M. hyosynoviae* associated lameness, the microorganism needs to be present in affected joints and synovial tissue must show characteristic histopathological lesions. Therefore, synovia should be aseptically collected from affected joints of alive or recently euthanized animals with a sterile syringe and an appropriately sized needle. Sedation of alive pigs is recommended to ease discomfort. Alternatively swabs and scrapings can be taken from joint cavities at necropsy after opening the joint under aseptic conditions. *M. hyosynoviae* affected joints tend to contain an increased volume of yellow to brown discolored synovia. The properties can be described as viscous with a serofibrinous or seroanguineous nature. Edematous, proliferative, or hyperemic synovial membranes which are dark in color are also suggestive for infections with this pathogen (Gomes Neto et al. 2012, Kobisch and Friis 1996, Nielsen et al. 2001). However, other microorganisms, e.g., *Glaesserella parasuis, Streptococcus suis, Erysipelothrix rhusiopathiae* and *M. hyorhinis* can cause similar pathological alterations in pig joints (Thacker 2004). For herd screenings and determination of the prevalence of *M. hyosynoviae,* tonsil scrapings (Gomes Neto et al. 2012) and pen based oral fluids (Pillman et al. 2019) can be used for pathogen detection.

The isolation and cultivation of *M. hyosynoviae* is rather challenging. Even when all sampling and sample preparation steps have been performed with caution, isolation results from affected joints are often disappointing. It seems that the agent is regularly present in concentrations below the detection limit or even absent during chronic or subacute infections phases. However, tenfold dilutions of joint samples taken from acutely affected joints should be used for cultivation. The dilution step is necessary because cell debris can inhibit the growth of this pathogen. If viable cells are present in sufficient numbers in the sample being tested the microorganism grows well on and in modified Hayflick's medium supplemented with mucinous material (Kobisch and Friis 1996). It is advised to use the same cultivation procedure for the isolation of *M. hyosynoviae* from tonsil scrapings. Uniformly formed colonies exhibiting fried egg morphology and a color change of agar and broth medium to red (pH shift to alkaline due to the release of ammonia from arginine hydrolysis) are suggestive for the cultivation of *M. hyosynoviae*. However, further tests are required for the final identification of the cultured mycoplasmas. Antigenic or genetic identification (Gomes Neto et al. 2012, Johansson et al. 1998, McAuliffe et al. 2005, Nathues et al. 2011, Poveda and Nicholas 1998, Stakenborg et al. 2006, Volokhov et al. 2012), as well as matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry (MS) (Spergser et al. 2019) are suited for this requirement.

Real time PCR is the most sensitive assay for the detection of *M. hyosynoviae* and is usually performed directly on clinical specimens (Gomes Neto et al. 2012, Gomes Neto et al. 2015, Pillman et al. 2019, Roos et al. 2019). Gomes Neto et al. (2016) demonstrated that different strains of *M. hyosynoviae* can vary in the duration of joint infections, so molecular diagnosis of *M. hyosynoviae* associated arthritis may depend on the strain involved. Hence, varying molecular detection results can be partially attributed to timing of necropsy related to the onset of clinical disease and to a low or absent bacterial load in joints at the time of necropsy. To secure the diagnosis of *M. hyosynoviae* associated arthritis, certain histopathological alterations must be present. These include hyperplasia of the synovial membrane, synovial villous hypertrophy, edema, mononuclear cell infiltration in the synovial subintima and mild fibrosis of the capsular (Gomes Neto et al. 2012, Macedo et al. 2019). Some field and experimental studies have shown promising test results when serodiagnosis of *M. hyosynoviae* infections and carriership has been applied (Gomes Neto et al. 2014, Nielsen et al. 2005, Zimmermann and Ross 1982). However, currently there are no validated immunoassays available for routine diagnostic purposes.

Due to the lack of a cell wall, *M. hyosynoviae* shows an intrinsic resistance to beta-lactam antibiotics which target the peptidoglycan layer of the cell wall. In addition, antibiotics which interfere with the folate synthesis (e.g., sulfonamides, trimethoprim, and combinations) have shown to have no bacteriostatic effect on this pathogen. It is therefore speculated that *M. hyosynoviae* lacks the folate synthesis pathway (Schultz et al. 2012). *M. hyosynoviae* isolates have shown *in vitro* sensitivity to tetracycline, tiamulin and enrofloxacin (Nielsen et al. 2001). More recent studies suggest likewise sensitivity to tiamulin and enrofloxacin, and in addition, to valnemulin, gentamicin and danofloxacin (Gautier-Bouchardon 2018), as well as to tylvalosin, tylosin and tilmicosin (Rosales et al. 2020). For the treatment of *M. hyosynoviae* associated arthritis, lincomycin delivered *via* the feed or water is one of the most commonly used antibiotics (Schmitt 2014). However, Rosales et al. (2020) have recently shown decreased susceptibility to lincomycin in field isolates circulating in Southern Europe. For this reason, the use of lincomycin should be more carefully evaluated. It needs to be noted that variable ranges of antibiotic susceptibility have been observed among different *M. hyosynoviae* strains. Some isolates were overall more resistant while others appeared to be more susceptible (Schultz et al. 2012).

Up to this date no commercial vaccines against *M. hyosynoviae* are available. Some manufacturers and producers have tested autogenous vaccines in field trials, but data on the outcome of these attempts are scarcely available (Schmitt 2014). Lauritsen et al. (2013) reported on the use of an autogenous vaccine that contained inactivated *M. hyosynoviae* cells and an adjuvant. Despite promising results under experimental conditions, this autogenous vaccine was not able to significantly lower the prevalence of lameness presumably caused by *M. hyosynoviae* in vaccinated pigs compared to the control group.

Typing techniques for porcine mycoplasmas

Musser (1996) highlighted the importance of precise molecular pathogen analysis to gain better insight on host pathogen interactions. The knowledge gained can then be used for disease prevention, control and outbreak investigations.

In the past, several molecular typing tools have been used for genetic characterization of porcine mycoplasmas, such as amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD) analysis, pulsed field gel electrophoresis (PFGE), multi-locus variable number of tandem repeats (VNTR) analysis (MLVA), and multi locus sequence typing (MLST). However, only few of these typing techniques have been used to analyze *M.*

hyosynoviae but were more widely applied to characterize strains from other porcine *Mycoplasma* species including *M. hyopneumoniae, M. hyorhinis, and M. flocculare*.

Kokotovic et al. (2002) reported the use of AFLP to determine the genetic relatedness of *M. hyosynoviae* isolates in Denmark. AFLP is a PCR based technique where DNA is digested with restriction enzymes and ligated with oligonucleotide adapters. Subsequently, the sets of fragmented DNA are amplified by PCR and visualized by a gel electrophoresis. Hereby, DNA fingerprinting is achieved without exact knowledge of nucleotide sequences (Vos et al. 1995).

RAPD is another PCR based typing technique first described in 1990 (Welsh and McClelland 1990, Williams et al. 1990). Targetless short primers with a random nucleotide pattern (approximately 10 base pairs (bp) long) are used to indiscriminately amplify chromosomal DNA by PCR. Different strains exhibit different fragment patterns shown by gel electrophoresis. RAPD has previously been applied for typing *M. hyopneumoniae* (Nathues et al. 2011, Vicca et al. 2003) but has never been used for the characterization of *M. hyosynoviae* strains.

PFGE has been developed in the early 1980s by Schwartz and Cantor (1984) and has been used for genotyping porcine mycoplasma strains. With this method large DNA fragments (>20 kb) produced by rare-cutting restriction enzymes are separated in an agarose gel where alternately pulsed and perpendicularly orientated electrical fields are applied. This periodical change of orientation of the electrical field is the key for the separation of large DNA fragments. PFGE is considered to be highly reproducible and can be theoretically applied on any organism (Mahon and Lehman 2019). PFGE has shown to be highly discriminatory in differentiating *M. hyopneumoniae* strains (Stakenborg et al. 2005) and has lately been used for the detection of genetic differences among *M. hyorhinis* isolates originating from specimens of pigs with respiratory disorders (Yamaguti et al. 2015).

MLVA is a typing technique targeting repetitive DNA sequences that vary in copy number (VNTR) which have been described for a variety of microorganisms. With MLVA several loci containing VNTRs are amplified by PCR and the number of repeats is then calculated from the PCR amplicon size (Mahon and Lehman 2019). MLVA has been successfully used for the molecular typing of *M. hyopneumoniae* isolates (Castro et al. 2006) and has also been applied on porcine bronchioalveolar lavage fluids and tracheal swabs without prior cultivation of the pathogen (Vranckx et al. 2011). MLVA has also been used to investigate the genetic relatedness of *M. hyopneumoniae* strains circulating in Serbia (Savic et al. 2010), Germany (Nathues et al. 2011), the United States of America, Brazil, Mexico, and Spain (Dos Santos,

Sreevatsan et al. 2015) as well as in Hungary, Czech Republic and Slovakia (Felde et al. 2018). MLVA has also shown differences among *M. hyopneumoniae* strains present within a production flow (Betlach et al. 2020) and within individual animals (Tonni et al. 2021). In addition, MLVA has also been applied for the molecular typing of *M. hyorhinis* (Dos Santos, Clavijo et al. 2015, Földi et al. 2020).

MLST is a derivation of multi locus enzyme electrophoresis and was developed and first used on *Neisseria meningitidis* (Maiden et al. 1998). MLST is used to characterize isolates of bacterial species by comparing sequences of internal fragments of housekeeping genes and different sequences present within each locus are assigned as distinct allele numbers. In most MLST schemes seven loci are indexed, but this number can be adjusted if a higher or lower resolution for typing is required. Nevertheless, all designations of the assessed loci are incorporated into an allelic profile or are assigned to a sequence type, so they can be stored in a database and used for comparison (Maiden et al. 2013). Overall, MLST has shown to provide reproducible, portable and comparable typing results that can be used for epidemiologic investigations (Mahon and Lehman 2019). Due to the global recognition of this method for accurate strain typing, a publicly accessible database (https://pubmlst.org/; Jolley et al. 2018) has been established housing thousands of alleles, allelic profiles and sequence types of >100 microbial species.

MLST has been widely used for molecular typing of *M. hyopneumoniae* (Balestrin et al. 2019, Felde et al. 2018, Kuhnert and Overesch 2014, Mayor et al. 2008, Overesch and Kuhnert 2017) and *M. hyorhinis* (Földi et al. 2020, Tocqueville et al. 2014, Trüeb et al. 2016). In addition, a MLST-s typing scheme has been described for *M. hyorhinis* in which two surface protein encoding genes next to two housekeeping genes have been included to improve discriminatory power (Clavijo et al. 2019). Up to date, no MLST scheme has been developed for genotyping *M. hyosynoviae* strains.

A novel typing technique for microbial pathogens including mycoplasmas is cgMLST. Schemes for *M. synoviae* (Ghanem and El-Gazzar 2018)*, M. gallisepticum* (Ghanem et al. 2018) and *M. anserisalpingitidis* (Kovács et al. 2020) has recently been described and one for *M. hyorhinis* (Bünger et al., submitted data) is currently in development. Core genome MLST combines the well-established MLST technique with whole genome sequencing. This technique offers a higher resolution than conventional MLST across a cluster of related but not identical strains. Through rapid and highly efficient sequencing methods, it is now possible to generate whole genome sequences of bacterial strains within a short period of time and for reasonable costs,

thus removing the practical constraints of previously designed approaches (Maiden et al. 2013). With cgMLST it is now feasible to evaluate and compare hundreds of core genome targets of sequenced genomes and therefore represents a novel tool for strain differentiation and epidemiological investigations. Like MLST, this technique provides high reproducibility, can be well standardized between different laboratories and offers an efficient way for strain typing of microorganisms within the same species (Ghanem et al. 2018, Neumann et al. 2019). An internet website (https://cgmlst.org/ncs) was created to share established cgMLST schemes globally.

3. Material and methods

Mycoplasma strains

In this diploma thesis a total of 51 *M. hyosynoviae* strains were investigated. Most of the isolates originated from Austrian domestic pigs and wild boars (n=28). *M. hyosynoviae* isolated from German pigs (n=23) provided by AniCon laboratory (AniCon Labor GmbH, Germany) were also included. 44 of the strains were recovered from domestic pigs (*Sus scrofa domesticus*) and seven isolates were cultured from clinical specimens derived from wild boars (*Sus scrofa)* during routine diagnostics at the Institute of Microbiology, University of Veterinary Medicine Vienna. All strains were isolated of samples derived from diseased animals between 2002 and 2021. After cultivation and identification to the species level through 16 rRNA gene sequencing (for isolates obtained prior to 2016) or MALDI-TOF MS (for isolates obtained after 2016) *M. hyosynoviae* strains were stored at - 80 °C until further investigation. The strains were labeled according to an internal number, sample type (L=lung, N=nasal cavity, J=joint, E=eye, T=trachea, LN=lymph node and S=serosa), year of isolation and county of origin (A=Austria and G=Germany). Isolates derived from samples of wild boars were additionally labeled with ‘ws’ (abbreviation for Wildschwein).

Additionally, nine publicly available genomes from strains isolated in Denmark, Canada and the USA were downloaded from the National Center for Biotechnology Information website and included in this work. The original strain designations were used to label these isolates.

Table 1: Metadata of *Mycoplasma hyosynoviae* strains used in this study.

Strain designation	Year	Host	Habitat	Geography	Farm	Used in cgMLST
L11N02A	2002	pig	nasal cavity	AUT		query genome
3138_3N02A	2002	pig	nasal cavity	AUT		
88N02A	2002	pig	nasal cavity	AUT		
1165L03A	2003	pig	lung	AUT		query genome
1737_1L03A	2003	pig	lung	AUT		
2056_4L03A	2003	pig	lung	AUT		
L30N03A	2003	pig	nasal cavity	AUT		
31J08A	2008	pig	joint	AUT		query genome
1854E08Aws	2008	wild boar	eye	AUT		
7_5L08A	2008	pig	lung	AUT		
2594J14A	2014	pig	joint	AUT		query genome
1140L15A	2015	pig	lung	AUT		query genome
3432_1J15G	2015	pig	joint	GER	G	
3432_2J15G	2015	pig	joint	GER	G	
3432_3J15G	2015	pig	joint	GER	G	query genome
3432_4J15G	2015	pig	joint	GER	G	
3432_6J15G	2015	pig	joint	GER	G	query genome
3517_18J15G	2015	pig	joint	GER	D	
986_1L16G	2016	pig	lung	GER	C	
986_2L16G	2016	pig	lung	GER	C	
986_3L16G	2016	pig	lung	GER	C	
986_4L16G	2016	pig	lung	GER	C	query genome
1997L16Aws	2016	wild boar	lung	AUT		
1792_3L16A	2016	pig	lung	AUT		
2788LN16Aws	2016	wild boar	lymph node	AUT		
3912L16Aws	2016	wild boar	lung	AUT		
171 9_741J17G	2017	pig	joint	GER	E	
216L19A	2019	pig	lung	AUT		
421hellL19A[#]	2019	pig	lung	AUT		
421dklL19A[#]	2019	Pig	lung	AUT		query genome
456J19G	2019	pig	joint	GER	F	
3156L19A	2019	pig	lung	AUT		
CB1132T19Aws	2019	wild boar	trachea	AUT		
VS1569T19ws	2019	wild boar	trachea	AUT		
4638L19A	2019	pig	lung	AUT		
425N20A	2020	pig	nasal cavity	AUT		query genome
1927L20A	2020	pig	lung	AUT		query genome
A1608804_007J20G	2020	pig	joint	GER	B	query genome
A1608804_006J20G	2020	pig	joint	GER	B	
A1608804_005J20G	2020	pig	joint	GER	B	
A1609339_004J20G	2020	pig	joint	GER	B	
A1921559_004J20G	2020	pig	joint	GER	A	
2481E20Aws	2020	wild boar	eye	AUT		
B1J20G	2020	pig	joint	GER	H	
B3J20G*	2020	pig	joint	GER	H	query genome

B4J20G*	2020	pig	joint	GER	H	
B5J20G*	2020	pig	joint	GER	H	
B7A1E20G§	2020	pig	eye	GER	H	
B7A2E20G§	2020	pig	eye	GER	H	
3718S20A	2020	pig	eye	AUT		
25N21A	2021	pig	nasal cavity	AUT		
ATCC 25591	<1970	pig	joint	USA		
M60	1968	pig	joint	DNK		seed genome
NPL1	2010	pig	joint	USA		
NPL2	2013	pig	joint	CAN		
NPL3	2012	pig	joint	USA		
NPL4	2010	pig	joint	USA		
NPL5	2012	pig	joint	USA		
NPL6	2011	pig	joint	USA		
NPL7	2013	pig	joint	USA		

AUT= Austria, GER= Germany, DNK= Denmark, CAN= Canada, USA= United States of America, #= Clonal isolates of one sample, the tested colonies showed a different morphological appearance on solid medium. *= Isolated from the same animal but from three different joints (B3J20G= tarsal, B4J20G= elbow and B5J20G= knee joint). §= Derived from an animal left and right eye.

Samples were stored at -80 °C. For re-cultivation purposes, 100 µl of each isolate were thawed and streaked onto SP4 agar plates which were then incubated for up to seven days at 37°C under 5% CO_2 atmosphere. A single colony of each isolate was then picked using a Pasteur pipette and further propagated in 3 ml SP4 broth medium at 37°C in ambient air until the late stage of logarithmic growth, which was indicated by a color change of the medium from red to a pink.

Matrix-assisted laser desorption/ionization – time of flight mass spectrometry

For species identification of colony-derived cultures, 1 ml of broth culture was centrifuged at 20.000 x g for seven min (Centrifuge 5424 R, Eppendorf AG, Germany). Afterwards, the supernatant was discarded, and the remaining pellet was resuspended with 100 µl of water (Water suitable for HPLC, Sigma-Aldrich Chemie GmbH, Germany) and centrifuged at 20.000 x g for five min. Again, the supernatant was removed, and proteins were extracted from the pellet by resuspension with 15 µl of 70% formic acid (ROTIPURAN® ≥98%, Carl Roth GmbH + Co. KG, Germany) and 15 µl of 100% acetonitrile (ROTISOLV®, Carl Roth GmbH + Co. KG, Germany). The sample was centrifuged again at 20.000 x g for two min.

For MALDI-ToF analysis, 1 µl of protein extract was spotted onto a MALDI-ToF carrier plate (axcess® MSP 96 target polished steel BC, Bruker Daltonik GmbH, Germany), air-dried at

room temperature, and overlaid by 1 µl of matrix solution (Bruker Matrix HCCA; portioned, Bruker Daltonik GmbH, Germany). After a further air-drying step, the samples were analyzed using a Bruker Biotyper (microflex® LRF, Bruker Daltonik GmbH, Germany) and FlexControl 3.4 software (Bruker Daltonik GmbH, Germany). Generated spectra were then compared to reference spectra stored in a large in-house mycoplasma database using MBT Compass & MBT Explorer software (Bruker Daltonik GmbH, Germany) and spectral concordance expressed by log score values were documented (Spergser et al. 2019).

After all colony-derived cultures had successfully been identified as *M. hyosynoviae*, 1 ml of each culture was centrifuged at 20.000 x g for seven min. The resulting pellet was then stored at -20 °C until further investigation.

DNA extraction and whole genome sequencing

A DNA extraction kit (MagAttract® HMW DNA, Qiagen N.V., Netherlands) was used to extract high molecular weight DNA. For this purpose, frozen bacterial pellets were thawed at room temperature for 15 min and resuspended with 180 µl of buffer ATL. After adding 20 µl proteinase K samples were incubated for 30 min at 56°C and approximately 700 rounds per minute (rpm) in a thermomixer (Eppendorf Thermomixer 5436, Eppendorf AG, Germany). The mixture was then allowed to cool at room temperature for five min. Four µl of RNase A were added and the samples were briefly mixed several times using a pulse-vortex mixer (Minishaker MS1, IKA Werke GmbH & Co. KG, Germany), and then incubated for two min at room temperature. Next, 15 µl of MagAttract Suspension G and 280 µl of Buffer MB were added. The samples were then vortexed and eventually mixed using a thermomixer at room temperature (1000 rpm) for three min.

Afterwards, the samples were placed on a magnetic rack (MagJET Separation Rack MR02, Thermo Fisher Scientific Inc., United States of America). After the magnetic beads had been separated from the liquid (usually after one minute), the supernatant was removed. After each following washing step, the supernatant was removed in the same manner.

For the first washing step, 700 µl of buffer MW1 were added to the magnetic beads. Then, the samples were incubated in a thermomixer at room temperature (1000 rpm) for one min. Next, samples were placed on the magnetic rack. After the bead separation was completed, the supernatant was removed again. This step was repeated once.

For the second washing step, 700 µl of Buffer PE were added to the samples. This was followed by another incubation step in a thermomixer (1000 rpm) at room temperature for one min. The magnetic beads were separated from the liquid and the supernatant was removed again. This step was repeated once as well.

For the third and final washing step, samples were left on the magnetic rack so that the magnetic beads remained fixed to the side of the tube. The magnetic bead containing the DNA were then rinsed with 700 µl of distilled water and incubated for one min and the supernatant was discarded again. This final washing step was repeated once.

Finally, samples were removed from the magnetic rack and 100 µl elution buffer AE were added. Samples were incubated at room temperature and 1000 rpm for three min. Samples were then placed on the magnetic rack and the magnetic bead were separated from the eluate containing high molecular weight DNA which was stored at -20 °C until send to AGES (Agentur für Gesundheit und Ernährungssicherheit GmbH, Austria) for whole genome sequencing.

Whole genomes sequencing was performed on ready-to-sequence libraries prepared by Nextera XT DNA Library Preparation Kit (Illumina Inc., USA). For paired end sequencing the Illumina MiSeq platform (Illumina Inc., USA) was used. SPAdes 3.10. with default parameters was applied for quality checking, trimming and de novo assembling of raw reads (Bankevich et al. 2012).

Core genome multi-locus sequence typing

Ridom® SeqSphere+ version 7.0 (Ridom GmbH, Germany) was used to develop a cgMLST scheme for *M. hyosynoviae*, in an equal manner as previously described for other mycoplasmas. First, the seed genome of *M. hyosynoviae* strain M60 which was the only completed genome available and 13 *M. hyosynoviae* query genomes were used to develop an *ad hoc* cgMLST scheme. Query genomes were selected based on cgMLST analysis of all *M. hyosynoviae* genomes used in this study blasted against genes of the reference genome identified after filtration. Subsequently, a minimum spanning tree (MST) and a neighbor joining tree (NJT) were generated, thereby identifying the relatedness among the tested strains. *M. hyosynoviae* strains with more than 100 alleles difference to their closest relatives and thus representing the overall diversity of the tested population, were selected as query genomes to increase the robustness of the developed cgMLST scheme. For cgMLST development, an appropriate set of target genes for cgMLST was defined, excluding all unfit gene targets. To

filter the reference genome for this purpose, the cgMLST definer tool of SeqSphere+ with default parameters was used. The following filters were applied: 1) a homologous gene filter, which excluded genes with more than 90% identity and more than 100 bp overlap, 2) a start codon filter, which excluded genes without a start codon, 3) a minimum length filter, which excluded genes with less than 50 bp, 4) a stop codon filter, which excluded genes with a missing stop codon, multiple stop codons and genes with a misplaced stop codon and 5) an overlap filter, which excluded the shorter of two genes that overlapped by more than four bp. Afterwards, the 13 query genomes were compared pairwise to select shared fit target genes by blasting them against the reference genomes target gene set. All reference genome-filtered genes that were found in all query genomes with a sequence identify of > 90% and 100% overlap formed the final targets of the cgMLST scheme. To evaluate the created cgMLST scheme, the remaining 60 *M. hyosynoviae* genomes were used for identification of cgMLST targets present in the *M. hyosynoviae* strain cohort. Finally, all 60 *M. hyosynoviae* isolates were analyzed using the newly established cgMLST scheme to identify individual allelic profiles applied to assemble a minimum spanning tree and a neighbor joining tree by pairwise ignoring missing values.

Table 2: Publicly available *Mycoplasma hyosynoviae* genomes additionally used for cgMLST scheme development.

Strain designation	Genome size (Mb)	Acsession Number	Sequencing technology
ATCC 25591	0.870663	SOCH00000000	Illumina HiSeq
M60	0.863547	CP008748	PacBio
NPL1	0.888107	JFKL00000000	Illumina MiSeq
NPL2	0.879068	JFKK00000000	Illumina MiSeq
NPL3	0.866715	JFKJ00000000	Illumina MiSeq
NPL4	0.892195	JFKI00000000	Illumina MiSeq
NPL5	0.864738	JFKM00000000	Illumina MiSeq
NPL6	0.850366	JFKH00000000	Illumina MiSeq
NPL7	0.848102	JFKG00000000	Illumina MiSeq

Mb= mega bases

4. Results

Cultivation, identification, and DNA extraction

All 51 *M. hyosynoviae* colony-derived cultures were grown within seven days in SP4 medium and were afterwards identified as *M. hyosynoviae* by MALDI-TOF MS. DNA was successfully extraction from the investigated strains.

Development and evaluation of the *Mycoplasma hyosynoviae* core genome multi locus sequence typing scheme

Whole genome sequences (WGS) of 51 *M. hyosynoviae* strains isolated from Austrian and German pigs were obtained by Illumina MiSeq sequencing. The remaining WGS were downloaded from the gen bank database of the National Center for Biotechnology Information (NCBI). For generating a reference task template, the genome of the strain M60 was used. After filtering the genes, a total of 584 were retained. Strain M60 was used as seed genome and 13 WGS of isolates (1140L15A, 1165L03A, 1927L20A, 2594J14A, 31J08A, 3432_3J15G, 3432_6J15G, 421dklL19A, 425N20A, 986_4L16G, A1608804_007J20G, B3J20G and L11N02A) were used as query genomes. Then a cgMLST scheme was defined consisting of 373 target genes (429,533 bases) covering 49.7% of the seed genome sequence. Accessory genes (n=174, 241,764 bp, 28% of the seed genome) were determined as well. The defined core target genes can be found in Table 3. To evaluate the created cgMLST scheme, WGS of the 60 included *M. hyosynoviae* isolates were loaded into SeqSphere+ and were typed accordingly. In all tested WGS at least 91,42% (mean 98.09% ± 1.59 standard deviation) good cgMLST target genes were found. The average number of alleles for 373 targets was 19 alleles (range three to 49 alleles). Strains with a maximum of seven alleles differences were grouped together as clonal clusters, thus representing a high degree of relatedness. Based on this premise, seven different clusters compromising 19 isolates were identified, but one of these clusters was formed by two strains for which genome data were downloaded from the NCBI (cluster 7; NPL3 and NPL5). Furthermore, strains with up to 89 alleles difference were considered as closely related based on their epidemiological background.

Core genome multi locus sequence typing of *Mycoplasma hyosynoviae* isolates

To test the developed typing scheme, cgMLST was performed on all 60 *M. hyosynoviae* strains included in this study.

Overall, epidemiological unrelated tested strains showed a high degree of diversity, which is shown by a high number of allele differences between them.

A clear separation between strains with publicly available genomes and the Austrian and German strain cohort was not apparent. However, the strains originating from the United States of America (NPL1 to NPL7 and ATCC25591) are grouped together in the MST but do not show an exceptional high number of allele differences compared to other tested strains. Furthermore, all strains from the USA share the same common ancestor in the NJT and are therefore branched early compared to the other tested stains. However, strain A1608804_005J20G and 1997L16Aws share the same common ancestor in the NJT even though they were isolated in Europe.

Only four strains with publicly available genome data (NPL3, NPL5, NPL6 and NPL7) clustered closely together and showed at best 22 alleles difference, furthermore strain NPL3 and NPL5 formed cluster 7, with no allele difference to each other.

Strains isolated from wild boars intermingled with other tested strains, thus no explicit wild boar cluster was evident. Three wild boar-derived strains (VS1569T19Aws, 2481E20Aws and CB1132T19Aws), however, shared a common ancestor in the NJT.

Nevertheless, epidemiologically related strains (i.e., strains isolated from individual animals or from different animals of one farm) grouped together or were even part of clonal clusters. For example, cluster 3 contains all four strains (986_1L16G, 986_2L16G, 986_3L16G and 986_4L16G) isolated from the lungs of pigs kept in one farm.

Cluster 5 (A1609339_004J20G and A1608804_006J20G) and cluster 6 (3432_1J15G, 3432_2J15G, 3432_3J15G and 3432_6J15G) each consists of strains derived from animals of a distinct farm, but certain strains (A1608804_005 and A1608804_007J20G for cluster 5; 3432_4J15G for cluster 6), despite the same origin showed high numbers of alleles differences in comparison to the grouped strains.

Strains of cluster 1 (B3J20G, B4J20G and B5J20G), cluster 2 (B7A1E20G and B7A2E20G) and isolate B1J20G were derived from three different animals of the same farm and are genetically closely related. Strain B1J20G, derived from a joint sample, exhibits less allelic

differences to cluster 2 than to cluster 1, although the isolates in cluster 1 were also derived from joint samples and not from eye samples, as was the case with the strains in cluster 2.

Cluster 4 consists of two isolates (421hellL19A and 421dklL19A) both derived from the lung of one animal. On solid medium, the two strains exhibited a different colony morphological appearance in respect of opacity, but now show only one allelic difference.

Overall, cgMLST typed the 60 *M. hyosynoviae* isolates used into 48 distinct sequence types (STs) based on more than seven allelic differences (threshold for defining a clonal cluster), resulting in a Simpson's index of diversity of 0.997. Excel (Microsoft Excel 2019, Microsoft Corporation, United States of America) and the equation shown in Figure 1 (Hunter and Gaston 1988) was used to calculate the index.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j-1)$$

Figure 1: Equation proposed by Hunter and Gaston (1988) to calculate Simpson's index of diversity.

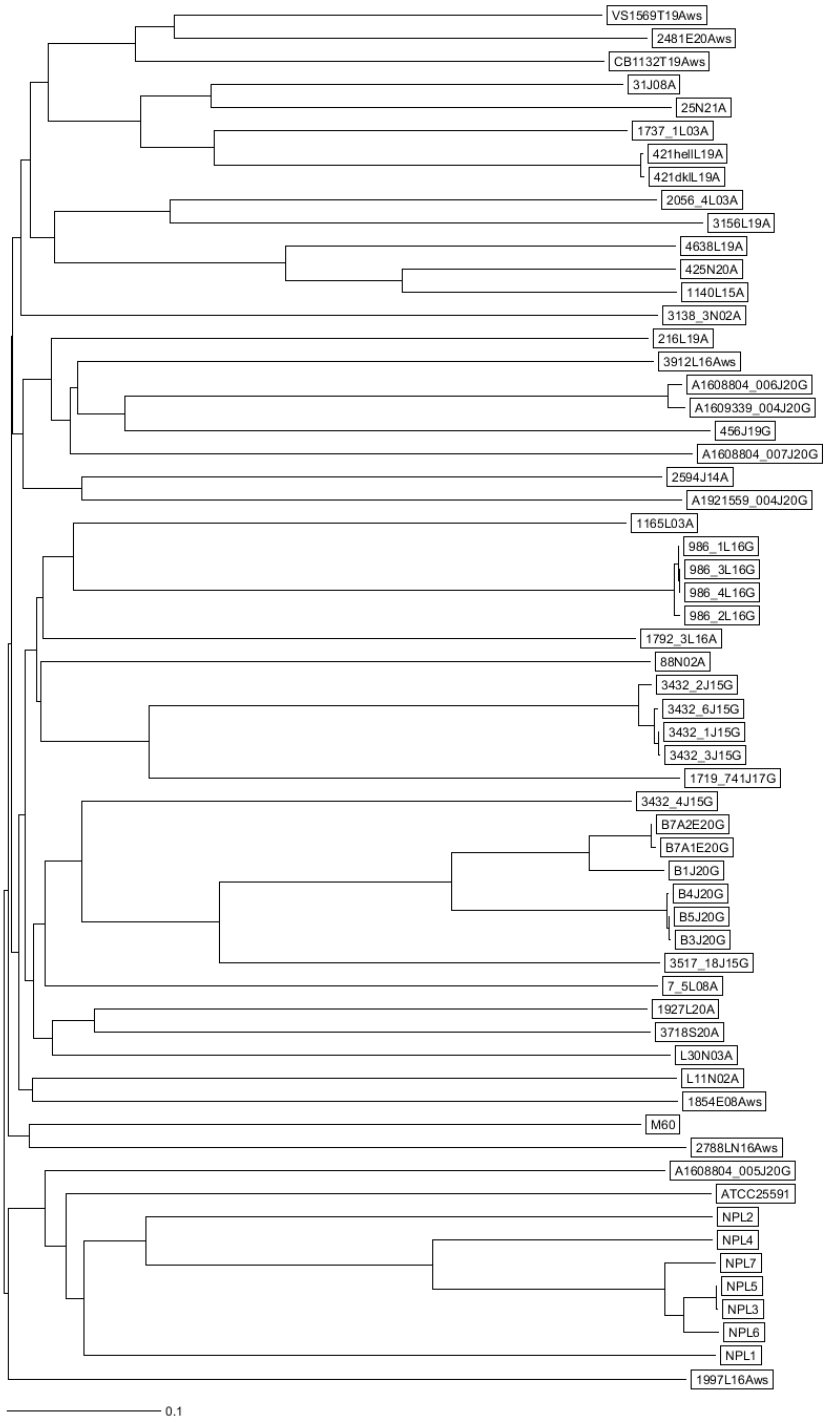

Figure 2: Neighbor joining tree of the 60 *Mycoplasma hyosynoviae* strains tested by cgMLST.

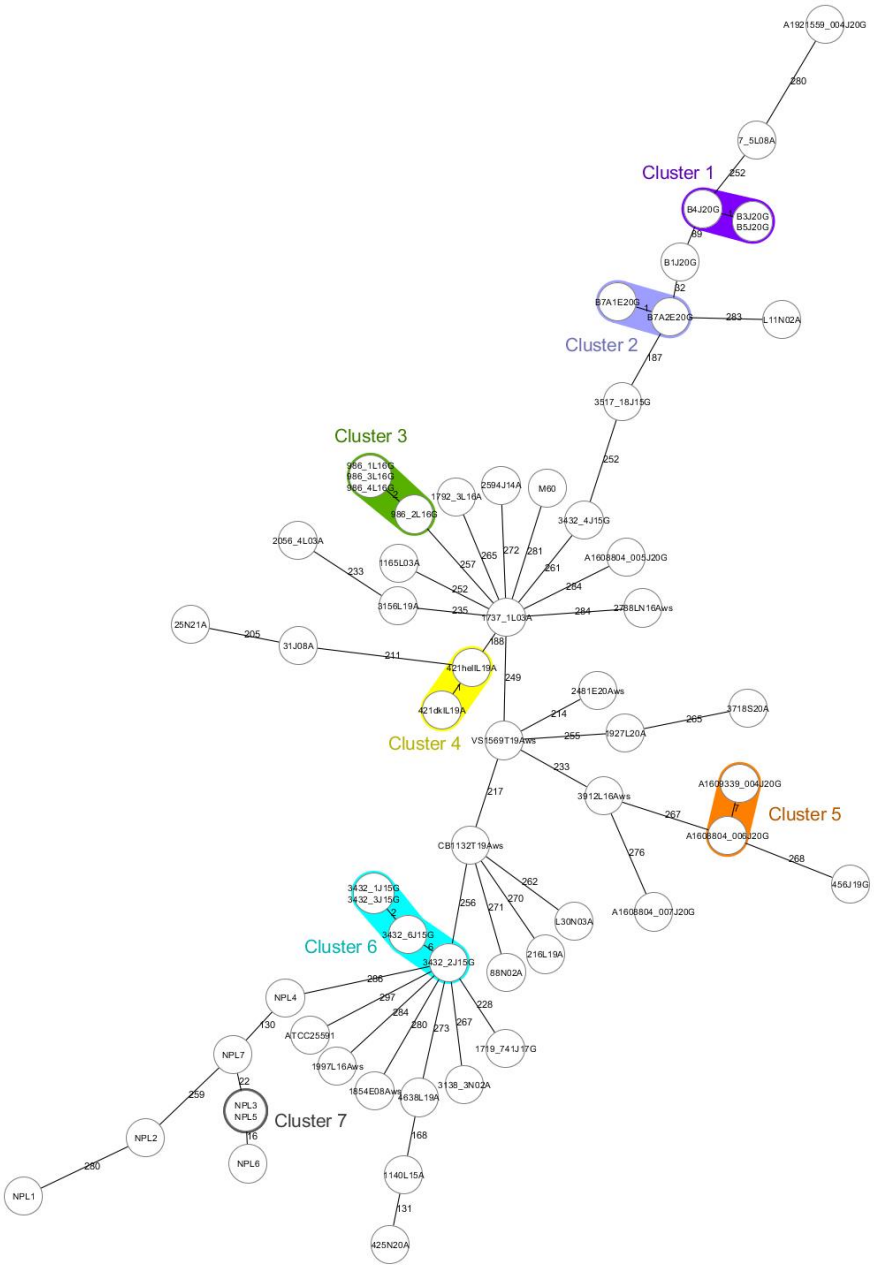

Figure 3: Minimum spanning tree of the 60 *Mycoplasma hyosynoviae* strains. The numbers above the corresponding connecting lines shows the numbers of different alleles between stains. Closely related isolates with less than seven alleles difference are grouped together in distinct clonal clusters, which are indicated by a colored border around them.

Table 3: The 373 core target genes used in cgMLST for the typing of *M. hyosynoviae* strains.

Target	GenBank product	Target	GenBank product	Target	GenBank product
MHSN_00005	chromosomal replication initiation protein	MHSN_01030	ATP synthase subunit gamma	MHSN_02440	hypothetical protein
MHSN_00010	DNA polymerase III subunit beta	MHSN_01035	ATP F0F1 synthase subunit beta	MHSN_02445	hypothetical protein
MHSN_00015	RNA-binding protein	MHSN_01070	hypothetical protein	MHSN_02450	S-adenosylmethionine synthase
MHSN_00020	50S ribosomal protein L28	MHSN_01075	hypothetical protein	MHSN_02455	aspartate--ammonia ligase
MHSN_00025	isochorismatase	MHSN_01080	ATPase AAA	MHSN_02470	phosphoglycerate kinase
MHSN_00030	alanine racemase	MHSN_01085	phenylalanyl-tRNA synthetase subunit alpha	MHSN_02475	hypothetical protein
MHSN_00050	hypothetical protein	MHSN_01090	phenylalanyl-tRNA synthetase subunit beta (pheT)	MHSN_02480	hypothetical protein
MHSN_00060	DNA gyrase subunit A	MHSN_01095	recombinase RecA	MHSN_02485	hypothetical protein
MHSN_00065	arginine deiminase	MHSN_01100	metallophosphatase	MHSN_02490	hypothetical protein
MHSN_00070	hypothetical protein	MHSN_01110	SAM-dependent methlyltransferase	MHSN_02495	elongation factor Tu
MHSN_00080	hypothetical protein	MHSN_01115	nitrogen fixation protein NifS	MHSN_02510	glycyl-tRNA ligase
MHSN_00100	hypothetical protein	MHSN_01125	hypothetical protein	MHSN_02520	RNA polymerase sigma factor
MHSN_00105	hypothetical protein	MHSN_01130	nicotinate-nucleotide adenylyltransferase	MHSN_02560	hypothetical protein
MHSN_00115	hypothetical protein	MHSN_01140	50S ribosomal protein L35	MHSN_02565	hypothetical protein
MHSN_00125	DNA ligase	MHSN_01145	50S ribosomal protein L20	MHSN_02575	single-stranded DNA-binding protein
MHSN_00130	cobalt ABC transporter permease	MHSN_01155	hypothetical protein	MHSN_02580	hypothetical protein
MHSN_00135	cobalt transporter ATP-binding subunit	MHSN_01175	hypothetical protein	MHSN_02585	preprotein translocase subunit SecG
MHSN_00145	antitermination protein NusG	MHSN_01180	ATP F0F1 synthase subunit alpha	MHSN_02595	histidyl-tRNA synthetase
MHSN_00155	hypothetical protein	MHSN_01185	ATP synthase F0F1 subunit beta	MHSN_02605	ATP-dependent DNA helicase RuvB

MHSN_00160	thiamine biosynthesis protein ThiI	MHSN_01190	hypothetical protein	MHSN_02615	inorganic pyrophosphatase
MHSN_00170	hypothetical protein	MHSN_01195	amino acid permease	MHSN_02620	hypothetical protein
MHSN_00175	hypothetical protein	MHSN_01200	hydrolase TatD	MHSN_02630	hypothetical protein
MHSN_00185	hydrolase	MHSN_01220	ABC transporter ATP-binding protein	MHSN_02635	hypothetical protein
MHSN_00220	pyruvate kinase	MHSN_01245	cation-transporting ATPase	MHSN_02640	elongation factor P
MHSN_00250	hypothetical protein	MHSN_01255	ribose-phosphate pyrophosphokinase	MHSN_02650	hypothetical protein
MHSN_00255	hypothetical protein	MHSN_01265	hypothetical protein	MHSN_02655	pseudouridine synthase
MHSN_00265	hypothetical protein	MHSN_01270	carbamate kinase	MHSN_02665	hypothetical protein
MHSN_00270	hypothetical protein	MHSN_01275	ornithine carbamoyltransferase	MHSN_02670	hypothetical protein
MHSN_00300	hypothetical protein	MHSN_01285	phosphoglycerate mutase	MHSN_02675	acyl carrier protein
MHSN_00310	hypothetical protein	MHSN_01305	hypothetical protein	MHSN_02695	hypothetical protein
MHSN_00320	hypothetical protein	MHSN_01310	isoleucyl-tRNA synthetase	MHSN_02700	tRNA pseudouridine synthase B
MHSN_00330	hypothetical protein	MHSN_01330	hypothetical protein	MHSN_02705	haloacid dehalogenase
MHSN_00335	hypothetical protein	MHSN_01395	tryptophan synthase subunit beta	MHSN_02710	hypothetical protein
MHSN_00340	ABC transporter ATP-binding protein	MHSN_01405	arginyl-tRNA synthetase	MHSN_02715	30S ribosomal protein S15
MHSN_00345	lactate dehydrogenase	MHSN_01410	cysteinyl-tRNA synthetase	MHSN_02720	50S ribosomal protein L21
MHSN_00350	hypothetical protein	MHSN_01420	phosphoesterase	MHSN_02730	hypothetical protein
MHSN_00375	protein kinase	MHSN_01435	hypothetical protein	MHSN_02735	tyrosyl-tRNA synthetase
MHSN_00400	GTPase	MHSN_01440	30S ribosomal protein S20	MHSN_02740	hypothetical protein
MHSN_00405	serine/threonine protein kinase	MHSN_01445	hypothetical protein	MHSN_02750	30S ribosomal protein S9
MHSN_00410	hypothetical protein	MHSN_01465	DNA gyrase subunit B	MHSN_02755	50S ribosomal protein L13
MHSN_00415	guanylate kinase	MHSN_01475	tRNA (guanine-N7)-methyltransferase	MHSN_02760	16S rRNA methyltransferase
MHSN_00420	DNA methylase	MHSN_01480	RNAase G	MHSN_02770	hypothetical protein

MHSN_00425	hypothetical protein	MHSN_01505	nicotinate phosphoribosyltransferase	MHSN_02780	pseudouridine synthase
MHSN_00430	hypothetical protein	MHSN_01510	hypothetical protein	MHSN_02785	proline dipeptidase
MHSN_00440	peptide ABC transporter permease	MHSN_01515	hypothetical protein	MHSN_02795	phosphoketolase
MHSN_00445	hypothetical protein	MHSN_01520	PAP phosphatase	MHSN_02800	50S ribosomal protein L31
MHSN_00455	hypothetical protein	MHSN_01530	glyceraldehyde-3-phosphate dehydrogenase	MHSN_02810	leucyl-tRNA synthetase
MHSN_00460	hypothetical protein	MHSN_01555	acetate kinase	MHSN_02825	mechanosensitive ion channel protein MscL
MHSN_00465	hypothetical protein	MHSN_01565	multidrug ABC transporter	MHSN_02830	hypothetical protein
MHSN_00475	ATP-dependent DNA helicase	MHSN_01580	30S ribosomal protein S4	MHSN_02850	excinuclease ABC subunit A
MHSN_00485	hypothetical protein	MHSN_01615	50S ribosomal protein L10	MHSN_02855	hypothetical protein
MHSN_00490	asparaginyl-tRNA synthetase	MHSN_01620	50S ribosomal protein L7/L12	MHSN_02860	hypothetical protein
MHSN_00495	serine--tRNA ligase	MHSN_01625	DNA-directed RNA polymerase subunit beta	MHSN_02865	hypothetical protein
MHSN_00500	hypothetical protein	MHSN_01630	DNA-directed RNA polymerase subunit beta'	MHSN_02870	hypothetical protein
MHSN_00505	peptidyl-tRNA hydrolase	MHSN_01635	hypothetical protein	MHSN_02875	signal recognition particle
MHSN_00510	hypothetical protein	MHSN_01640	lactate dehydrogenase	MHSN_02920	GTPase
MHSN_00515	hypothetical protein	MHSN_01645	methionyl-tRNA synthetase	MHSN_02925	RNA methyltransferase
MHSN_00525	hypothetical protein	MHSN_01650	GTPase CgtA	MHSN_02935	tRNA (guanine-N1)-methyltransferase
MHSN_00530	glutamate--tRNA ligase	MHSN_01740	30S ribosomal protein S10	MHSN_02940	30S ribosomal protein S16
MHSN_00545	hypothetical protein	MHSN_01745	50S ribosomal protein L3	MHSN_02945	cytidine deaminase
MHSN_00550	hypothetical protein	MHSN_01750	50S ribosomal protein L4	MHSN_02955	hypothetical protein
MHSN_00555	peptidase M17	MHSN_01760	50S ribosomal protein L2	MHSN_02960	cysteine methyltransferase
MHSN_00560	peptidase M17	MHSN_01765	30S ribosomal protein S19	MHSN_02965	elongation factor G
MHSN_00570	methionyl-tRNA formyltransferase	MHSN_01775	30S ribosomal protein S3	MHSN_02970	30S ribosomal protein S7

MHSN_00585	tRNA modification GTPase TrmE	MHSN_01795	50S ribosomal protein L14	MHSN_02975	30S ribosomal protein S12
MHSN_00590	hypothetical protein	MHSN_01800	50S ribosomal protein L24	MHSN_02985	methionine sulfoxide reductase A
MHSN_00595	cell division protein FtsY	MHSN_01805	50S ribosomal protein L5	MHSN_02990	ribonuclease BN
MHSN_00605	uridylate kinase	MHSN_01810	30S ribosomal protein S8	MHSN_02995	glutamyl-tRNA amidotransferase
MHSN_00610	ribosome-recycling factor	MHSN_01815	50S ribosomal protein L6	MHSN_03005	hypothetical protein
MHSN_00615	hypothetical protein	MHSN_01820	50S ribosomal protein L18	MHSN_03010	pseudouridine synthase
MHSN_00625	membrane protein	MHSN_01825	30S ribosomal protein S5	MHSN_03025	acyl-phosphate glycerol 3-phosphate acyltransferase
MHSN_00630	serine hydroxymethyltransferase	MHSN_01830	50S ribosomal protein L15	MHSN_03030	hypothetical protein
MHSN_00640	dihydroxyacetone kinase	MHSN_01835	preprotein translocase subunit SecY	MHSN_03035	hypothetical protein
MHSN_00645	fatty acid/phospholipid synthesis protein	MHSN_01845	methionine aminopeptidase	MHSN_03040	hypothetical protein
MHSN_00655	malate permease	MHSN_01850	translation initiation factor IF-1	MHSN_03045	hypothetical protein
MHSN_00660	ABC transporter ATP-binding protein	MHSN_01855	30S ribosomal protein S13	MHSN_03050	hypothetical protein
MHSN_00665	hypothetical protein	MHSN_01860	30S ribosomal protein S11	MHSN_03055	ABC transporter ATP-binding protein
MHSN_00670	hypothetical protein	MHSN_01865	DNA-directed RNA polymerase subunit alpha	MHSN_03060	hypothetical protein
MHSN_00675	Virulence-associated protein D	MHSN_01870	50S ribosomal protein L17	MHSN_03075	hypothetical protein
MHSN_00680	hypothetical protein	MHSN_01875	50S ribosomal protein L32	MHSN_03080	membrane protein
MHSN_00685	excinuclease ABC subunit B	MHSN_01885	hypothetical protein	MHSN_03085	hypothetical protein
MHSN_00695	threonyl-tRNA synthase	MHSN_01990	hypothetical protein	MHSN_03090	GTP-binding protein LepA
MHSN_00700	tryptophan--tRNA ligase	MHSN_01995	hydrolase	MHSN_03095	peptide ABC transporter ATP-binding protein
MHSN_00705	PTS glucose transporter subunit IIB	MHSN_02000	hypothetical protein	MHSN_03100	peptide ABC transporter ATP-binding protein

MHSN_00715	peptide deformylase	MHSN_02005	deoxyribose-phosphate aldolase	MHSN_03105	hypothetical protein
MHSN_00730	single-stranded DNA-binding protein	MHSN_02010	thymidine phosphorylase	MHSN_03110	peptide ABC transporter permease
MHSN_00740	GTP-binding protein	MHSN_02015	purine nucleoside phosphorylase DeoD-type	MHSN_03125	hypothetical protein
MHSN_00745	hypothetical protein	MHSN_02020	hypothetical protein	MHSN_03135	hypothetical protein
MHSN_00760	ABC transporter permease	MHSN_02080	hypothetical protein	MHSN_03140	N(G),N(G)-dimethylarginine dimethylaminohydrolase
MHSN_00765	hypothetical protein	MHSN_02085	tRNA synthetase subunit beta	MHSN_03145	hypothetical protein
MHSN_00770	hypothetical protein	MHSN_02090	endonuclease IV	MHSN_03150	Xaa-Pro aminopeptidase
MHSN_00775	hypothetical protein	MHSN_02100	hypoxanthine phosphoribosyltransferase	MHSN_03155	50S ribosomal protein L33
MHSN_00780	ABC transporter substrate-binding protein	MHSN_02105	hypothetical protein	MHSN_03165	hypothetical protein
MHSN_00785	ABC transporter permease	MHSN_02125	hypothetical protein	MHSN_03170	hypothetical protein
MHSN_00790	peptide ABC transporter permease	MHSN_02135	GTP-binding protein Der	MHSN_03175	hypothetical protein
MHSN_00800	ABC transporter ATP-binding protein	MHSN_02145	hypothetical protein	MHSN_03180	transcriptional regulator
MHSN_00805	ABC transporter permease	MHSN_02150	DNA topoisomerase I	MHSN_03200	hypothetical protein
MHSN_00850	ribose 5-phosphate isomerase	MHSN_02155	molecular chaperone DnaJ	MHSN_03205	hypothetical protein
MHSN_00855	SUA5-like translation suppressor	MHSN_02165	peptide chain release factor 1	MHSN_03215	hypothetical protein
MHSN_00860	hypothetical protein	MHSN_02220	hypothetical protein	MHSN_03220	hypothetical protein
MHSN_00870	hypothetical protein	MHSN_02235	hypothetical protein	MHSN_03225	hypothetical protein
MHSN_00885	spermidine/putrescine ABC transporter ATP-binding protein	MHSN_02240	hypothetical protein	MHSN_03235	glucose-inhibited division protein A
MHSN_00895	GTP-binding protein Era	MHSN_02265	MraZ family transcriptional regulator	MHSN_03245	translation initiation factor IF-2
MHSN_00900	hypothetical protein	MHSN_02270	16S rRNA methyltransferase	MHSN_03260	RNA-binding protein S1
MHSN_00905	hypothetical protein	MHSN_02275	hypothetical protein	MHSN_03265	hypothetical protein

MHSN_00910	magnesium ABC transporter ATPase	MHSN_02280	cell division protein FtsZ	MHSN_03270	FMN-dependent NADH-azoreductase
MHSN_00915	16S rRNA pseudouridylate synthase	MHSN_02285	hypothetical protein	MHSN_03275	50S ribosomal protein L1
MHSN_00925	hypothetical protein	MHSN_02305	hypothetical protein	MHSN_03280	50S ribosomal protein L11
MHSN_00930	DNA gyrase subunit B	MHSN_02310	hypothetical protein	MHSN_03285	hypothetical protein
MHSN_00935	licA	MHSN_02315	hypothetical protein	MHSN_03295	proline iminopeptidase
MHSN_00940	hypothetical protein	MHSN_02335	enolase	MHSN_03305	hypothetical protein
MHSN_00945	hypothetical protein	MHSN_02345	hypothetical protein	MHSN_03335	elongation factor Ts
MHSN_00950	lysyl-tRNA synthetase	MHSN_02350	preprotein translocase subunit SecA	MHSN_03345	molecular chaperone DnaK
MHSN_00955	hypothetical protein	MHSN_02355	hypothetical protein	MHSN_03355	HrcA family transcriptional regulator
MHSN_00960	hypothetical protein	MHSN_02360	hypothetical protein	MHSN_03385	hypothetical protein
MHSN_00965	haloacid dehalogenase	MHSN_02365	hypothetical protein	MHSN_03390	hypothetical protein
MHSN_00970	potassium transporter KtrA	MHSN_02380	hypothetical protein	MHSN_03400	alanyl-tRNA synthetase
MHSN_00980	potassium transporter KtrB	MHSN_02395	hypothetical protein	MHSN_03405	thiouridylase
MHSN_00985	hypothetical protein	MHSN_02405	peptidase	MHSN_03410	hypothetical protein
MHSN_00990	hypothetical protein	MHSN_02410	oligoendopeptidase F	MHSN_03420	hypothetical protein
MHSN_01000	hypothetical protein	MHSN_02415	adenine phosphoribosyltransferase	MHSN_03425	phosphopentomutase
MHSN_01005	ATP synthase F0F1 subunit A	MHSN_02420	sugar ABC transporter permease	MHSN_03440	thioredoxin
MHSN_01010	ATP synthase subunit C	MHSN_02430	sugar ABC transporter ATP-binding protein	MHSN_03455	hypothetical protein
MHSN_01015	ATP synthase subunit B	MHSN_02435	hypothetical protein	MHSN_03465	50S ribosomal protein L34
MHSN_01020	ATP synthase subunit delta				

5. Discussion

In this diploma thesis, the first whole genome sequence based cgMLST scheme was proposed for the use in *M. hyosynoviae* strain typing. The high discriminatory power of this method is demonstrated by the fact that it was possible to type the 60 included *M. hyosynoviae* strains into 48 different STs resulting in a Simpson's index of diversity of 0.997. Other authors (Ghanem et al. 2018, Ghanem and El-Gazzar 2018) reported a similar high discriminatory power of cgMLST schemes for other *Mycoplasma* species. The downside of cgMLST compared to other typing techniques is the higher monetary and labor costs. However, due to the rapid advances in next generation sequencing which is accompanied with cost reduction one downside can be lifted soon. In addition, there are efforts for an alternative sequencing approach that can be directly performed on clinical specimens, thus removing the necessity of the laborious and difficult cultivation of mycoplasmas (Ghanem et al. 2018).

The average number of found target genes for the tested 60 *M. hyosynoviae* strains was 98.09%. Been et al. (2015) reported that for a stable core genome at least 95% of its target genes should be found in any typed sample. However, in this study, four strains were found to have less than 95% core target genes (986_2L16G 94.91%, 3912L16Aws 94.64%, 3432_2J15G 91.42% and 1737_1L03A 92.49%). Strain 986_2L16G and 3912L16Aws narrowly missed the proposed threshold, but the gap was considerable larger for isolate 3432_2J15G and 1737_1L03A. In this study, 373 core target genomes were utilized, which is within the range of the number of core genomes used for development of other cgMLST schemes. For the cgMLST scheme of *M. synoviae* 302 (Ghanem and El-Gazzar 2018), for *M. gallisepticum* 425 (Ghanem et al. 2018) and for *M. anserisalpingitidis* 331 core genomes were used (Kovács et al. 2020). If this problem occurs more frequently with larger sampling groups, the selected core target genes may need to be reduced and reevaluated.

If strains had fewer than seven allele differences, they were considered as a distinct clonal cluster in this study. A similar threshold for clustering was chosen for *M. gallisepticum*, where isolates were considered as clonal clusters if fewer than ten differences were present (Ghanem et al. 2018). This threshold was intended to account for nucleotide variability within a strain that may occur after multiple passages (Ghanem and El-Gazzar 2018). For this thesis, the value for declaring strains distinct clusters was appointed based on cgMLST results. In the MST, there were a number of strains with up to seven alleles difference and a closely related epidemiological background. Therefore, these isolates were considered as clonally related strains. The next most closely related strains have at best 16 alleles difference (Cluster 7 to

strain NPL6), which is more than twice as much as the estimated value for clonal clusters and therefore were considered closely related. Strains with up to 89 alleles difference were also considered closely related (e.g., cluster 1 to strain B1J20G). Above this number there is a jump in allelic differences, so the threshold for related strains was set at 89. For solid evaluation of the clonal cluster threshold, a representative number of strains should be tested in between multiple passages, to get better insight on the allelic differences that might occur (Ghanem and El-Gazzar 2018).

Only strains originating from the same animal or from animals of a single farm grouped together and formed distinct clonal clusters. This is true for cluster 3 since all strains of one origin are included there. Cluster 5 and cluster 6 also contain most of the strains from the respective origin, but some exceptions are evident. Isolate A1608804_005J20G and A1608804_007J20G for cluster 5 and isolate 3432_4J15G for cluster 6 showed high allelic differences despite being derived from the same site as the other strains in their respective cluster. In addition, strain A1608804_005J20G and A1608804_007J20G (which originated from one farm) did not show a high degree of relatedness either, which may indicate that several different subpopulations of *M. hyosynoviae* can be present within a single farm. So far, this finding has not been described for *M. hyosynoviae* in the literature. However, these observations have been endorsed for *M. hyopneumoniae*, as different subpopulations of this pathogen can be present in a single farm, within an animal and even within a single organ (Betlach et al. 2020, Nathues et al. 2011, Tonni et al. 2021).

Furthermore, subpopulations of *M. hyosynoviae* with different degree of invasiveness can be derived from different anatomical location within animals of one farm. This phenomenon can be seen in cluster 1, cluster 2 and isolate B1J20G, which were cultured of clinical samples of a single farm but from different animals. The strains which form the clonal cluster 1 (B3J20G, B4J20G and B5J20G) were cultured from samples of three different joints (B3J20G= tarsal, B4J20G= elbow and B5J20G= knee joint) of a single animal. Also isolate B1J20G originates from a joint sample but from another pig. In addition, strain B7A1E20G and B7A1E20G, which form cluster 2, were cultured from eye samples taken from a single pig of the same farm. It is interesting to note that strain B1J20G is more closely related to cluster 1 and 2, than cluster 1 is to cluster 2. Therefore, it is speculated that there must be a certain difference in the genetic constitution of isolates within cluster 1 and strain B1J20G compared to cluster 2 that mediates clinical invasiveness, confer the ability to spread hematogenous and ultimately colonize and infect joints.

Strains derived from wild boar samples are not grouped together and intermingle with strains derived from domesticated pigs. Thus, it is not possible to identify a host-specific subpopulation of *M. hyosynoviae* isolates. In consequence, it is speculated that cross infection between domesticated pigs and wild boars with *M. hyosynoviae* may occur. However, up to this date, cases of *M. hyosynoviae* infection in domesticated pigs associated with transmission from wild boars have never been described. Also, data are missing on the prevalence of *M. hyosynoviae* in the wild boar population. However, current reports show that feral pigs may exhibit seroprevalence rates for *M. hyopneumoniae* from 19.7 up to 24.8% in Italy, Brazil, and Sweden, respectively (Bertelloni et al. 2020, Malmsten et al. 2018, Severo et al. 2021), and may therefore act as reservoir for this microorganism. However, due to the strict handling of free-range pigs (because of the threat of African swine fever in Europe) and the traditional housing conditions of swine in Europe it is unlikely that transmission of *M. hyosynoviae* and *M. hyopneumoniae* from wild boars to domesticated pigs may occur on a regular basis.

It should be noted that this study only includes strains derived from diseased animals. That was also the reason for further diagnostic testing, including detection of *M. hyosynoviae*. Therefore, the sampling method was not representative, and the results do not reflect the entire *M. hyosynoviae* population in the respective farms and the existing *M. hyosynoviae* population in Austria and Germany. For a better understanding of the epidemiology and the population structure of *M. hyosynoviae*, a more representative sample pool should be analyzed. This should include commensal strains derived from apparently unaffected animals and farms. In addition, more information regarding pig flow and transmission routes of the pathogen is desirable. Finally, as the current finding suggests that multiple subpopulations exist within a farm a larger sampling size per location should be evaluated.

6. Summary

Mycoplasma (*M.*) *hyosynoviae* is a swine-adapted pathogen of the class *Mollicutes*, which is often found as a commensal in the tonsils and the upper airways of the porcine host. The pathogen can also spread systemically, thereby causing non-purulent polyarthritis primarily in pigs at the end of the fattening period. The underlying pathomechanisms as well as the epidemiology of *M. hyosynoviae* infections are still largely unknown. Therefore, the aim of this diploma thesis was to establish a high-resolution method (core genome multi-locus sequence typing, cgMLST) for typing *M. hyosynoviae* strains to accurately determine the relationship between field isolates and to better understand the epidemiology of *M. hyosynoviae* infections.

Whole genome sequences obtained by Illumina sequencing (MiSeq) of 51 strains isolated from clinical samples of domestic pigs (n=44) and wild boars (n=7) from Austria (n=28) and Germany (n=23) as well as nine published whole genomes were used for the development, validation, and implementation of the presented cgMLST scheme. Using Ridom® SeqSphere+ software a cgMLST scheme comprising 373 core genome target genes was developed.

The developed cgMLST method demonstrated high discriminatory power as it was able to distinguish the 60 *M. hyosynoviae* strains examined into 48 different cgMLST sequence types resulting in a Simpson's index of diversity of 0.997. Seventeen *M. hyosynoviae* strains isolated from samples taken from five farms and from 13 animal were grouped into six clonal clusters (allelic difference ≤ 7). However, *M. hyosynoviae* strains from the same farm could also be distinguished as they exhibited a higher number of allele differences. Otherwise, investigated isolates from different farms showed a high degree of diversity expressing extensive allelic distances. Besides, it was not possible to identify a host-specific *M. hyosynoviae* population in wild boars.

In summary, the results of the thesis show that the newly developed cgMLST scheme is a highly discriminative tool for typing *M. hyosynoviae* strains and is therefore ideally suited for epidemiological studies including outbreak analyses.

7. Zusammenfassung

Mycoplasma (*M.*) *hyosynoviae* ist ein an das Schwein adaptierter Erreger aus der Klasse *Mollicutes*, welcher häufig als Kommensale in den Tonsillen und oberen Atemwegen seines Wirts nachzuweisen ist. Außerdem besitzt der Erreger die Fähigkeit sich systemisch auszubreiten und dabei nicht eitrige Polyarthritiden vorranging bei Schweinen am Ende der Mastperiode zu verursachen. Die zugrundeliegenden Pathomechanismen sowie die Epidemiologie von *M. hyosynoviae*-Infektionen sind jedoch bis heute kaum erforscht. Deshalb war es das Ziel dieser Diplomarbeit, ein hochauflösendes Verfahren (Kerngenom-Multilokus-Sequenztypisierung, cgMLST) zur Typisierung von *M. hyosynoviae*-Stämmen zu etablieren, um dabei die Verwandtschaftsverhältnisse von Feldisolate korrekt darstellen und die Epidemiologie der *M. hyosynoviae*-Infektion besser nachvollziehen zu können.

Für die Entwicklung, Validierung und Durchführung des cgMLST-Schemas wurden Gesamtgenome (Illumina MiSeq) von 51 Stämmen, die aus klinischen Proben von Hausschweinen (n=44) und Wildschweinen (n=7) aus Österreich (n=28) und Deutschland (n=23) isoliert werden konnten, sowie neun veröffentlichte Gesamtgenome verwendet. Mithilfe der Ridom® SeqSphere+ Software wurde ein 373 Zielgene-umfassendes cgMLST-Schema entwickelt.

Das entwickelte cgMLST-Verfahren wies eine hohe Trennschärfe auf, da es die 60 untersuchten *M. hyosynoviae*-Stämme in 48 cgMLST-Sequenztypen unterscheiden konnte, was zu einem Simpson's Index of Diversity von 0,997 führte. Siebzehn *M. hyosynoviae*-Isolate, die aus Proben von fünf Betrieben und insgesamt 13 Tieren stammten, konnten in sechs klonale Cluster (Alleldifferenz ≤ 7) gruppiert werden. Daneben konnten aber auch *M. hyosynoviae*-Stämme desselben Betriebs anhand der erhöhten Anzahl von Alleldifferenzen unterschieden werden. Ansonsten wiesen die untersuchten Isolate aus unterschiedlichen Betrieben eine hohe Diversität mit weitreichenden Alleldistanzen auf. Eine Wildschwein-spezifische Erregerpopulation konnte nicht identifiziert werden.

Zusammenfassend zeigten die Ergebnisse der Diplomarbeit auf, dass es sich bei dem neu entwickelten cgMLST-Schema um ein hochauflösendes Werkzeug für die Typisierung von *M. hyosynoviae*-Stämmen handelt und das Verfahren deshalb bestens für epidemiologische Untersuchungen und zur Aufklärung von komplexen epidemiologischen Fragestellungen (z.B. Ausbruchsanalysen) geeignet ist.

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9. List of figures and tables

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