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**Proteomic and genomic characterisation of *Mycoplasma felis*
isolated from cats, wild *Felinae* and horses**

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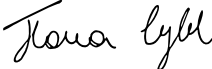
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ABSTRACT

Mycoplasma (M.) felis is a globally distributed, opportunistic pathogen primarily associated with infections in domestic cats. It has been implicated in various clinical manifestations including conjunctivitis, respiratory tract disease, arthritis, and serosal inflammation. Although commonly considered a commensal organism of feline mucosal surfaces, accumulating evidence highlights its pathogenic potential under certain host or environmental conditions.

This thesis investigated the relatedness of *M. felis* isolates obtained from domestic cats, wild *Felinae*, and horses. A total of 49 *M. felis* isolates including two different colony morphotypes (opaque, lucent) of seven isolates, and the type strain of *M. felis* (CO^T) were analyzed using genomic and proteomic typing methods. A newly established core genome multilocus sequence typing (cgMLST) scheme was employed to construct phylogenetic relationships among *M. felis* strains. For the proteomic characterization matrix assisted laser desorption ionization – time of flight (MALDI-ToF) mass spectrometry was used to generate protein mass spectra from which a dendrogram reflecting spectral distances was created.

The newly developed cgMLST scheme encompassing 454 target genes revealed high diversity of the studied *M. felis* strains separated into two major clusters, with some evidence of host-specific clustering of equine isolates. Interestingly, the colony variants of *M. felis* isolates (opaque or lucent) each clustered together or even showed an identical cgMLST profile, suggesting that the colony morphotype is driven by genes that are not part of the core genome. In contrast, although the MALDI-TOF mass spectrometry also grouped the isolates into two main clusters, no correlations were found between clustering and epidemiologic data or phylogenetic grouping based on cgMLST. Overall, the newly established cgMLST appears to be a suitable method for investigating the epidemiology of *M. felis* infection and gaining insights into the population structure of this underestimated pathogen.

ZUSAMMENFASSUNG

Mycoplasma (M.) felis ist ein weltweit verbreiteter, opportunistischer Erreger, der primär mit Infektionen bei Hauskatzen in Verbindung gebracht wird. Der Erreger wird mit verschiedenen klinischen Krankheitsbildern assoziiert, darunter Bindehautentzündung, Atemwegserkrankungen, Arthritis und Serositis. Obwohl *M. felis* häufig als harmloser Bewohner der Schleimhäute von Katzen angesehen wurde, gibt es zunehmend Hinweise auf sein pathogenes Potenzial.

In der vorliegenden Arbeit wurden die Verwandtschaftsverhältnisse zwischen *M. felis*-Isolaten untersucht, die von Hauskatzen, wilden Kleinkatzen (*Felinae*) und Pferden stammten. Insgesamt wurden 49 *M. felis*-Isolate, darunter zwei unterschiedliche Koloniemorphotypen (opak, transparent) von sieben Isolaten, und der Typstamm CO^T mittels genomischer und proteinanalytischer Typisierungsmethoden untersucht. Für die genotypische Charakterisierung wurde ein neuentwickeltes Kerngenom-Multilokus-Sequenztypisierungsschema (cgMLST) eingesetzt, um die phylogenetischen Verwandtschaftsbeziehungen zwischen den *M. felis*-Stämmen darzustellen. Für die proteinanalytische Charakterisierung wurde die Matrix-unterstützte Laser-Desorption-Ionisation mit Flugzeitanalyse (MALDI-ToF) Massenspektrometrie angewandt und Distanzwerte der dabei generierten Protein-Massenspektren als Dendrogramm abgebildet.

Mithilfe des neu etablierten cgMLST-Schemas, welches 454 Zielgene umfasste, konnte eine hohe Diversität der untersuchten *M. felis*-Stämme ermittelt werden, die in zwei Hauptcluster unterteilt wurden. Hinweise auf eine wirtsspezifische Gruppierung waren durch die Ausbildung eines aus equinen *M. felis*-Isolaten bestehenden Subclusters erkennbar. Interessanterweise gruppieren sich die Kolonievarianten von *M. felis*-Isolaten (opake oder durchscheinende Kolonien) jeweils zusammen oder zeigten sogar ein identes cgMLST Profil, was darauf hindeutet, dass der Koloniemorphotyp von Genen gesteuert wird, die nicht zum Kerngenom gehören. Im Gegensatz dazu gruppierte die MALDI-TOF-Massenspektrometrie die Isolate zwar auch in zwei Hauptcluster, es konnten jedoch keine Zusammenhänge zwischen der Clusterbildung und den epidemiologischen Daten sowie der phylogenetischen Gruppierung mittels cgMLST festgestellt werden. Insgesamt erscheint die neu etablierte cgMLST eine geeignete Methode zu sein, um die Epidemiologie von *M. felis*-Infektionen darzustellen und Einblicke in die Populationsstruktur dieses unterschätzten Erregers zu gewinnen.

LIST OF ABBREVIATIONS

BAL – bronchoalveolar lavage

cgMLST – core genome multilocus sequence typing

HGT – horizontal gene transfer

IAD – inflammatory airway disease

LRTD – lower respiratory tract disease

M. – *Mycoplasma*

MALDI-TOF MS – matrix assisted laser desorption ionization – time of flight

NCBI – National Center for Biotechnology Information

rpm – rounds per minute

URTD – upper respiratory tract disease

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1. Introduction

Mycoplasma (M.) felis is a widespread opportunistic pathogen associated with a variety of diseases in several host species across the globe. The most common hosts are domestic cats, although cases of *M. felis* infection in wild felids and horses have been reported. While the bacterium mainly resides in the respiratory tract, conjunctiva and the oropharynx, it has also been found in joints and lymphatic organs (1). This bacterium has primarily been associated with conjunctivitis, upper respiratory tract disease (URTD), lower respiratory tract disease (LRTD), and occasionally with pleuritis, pericarditis, and arthritis (2–7). While it was previously assumed that *M. felis* is a mere commensal belonging to the autochthonous microbiota of feline mucous membranes, these claims have been discussed controversially. Even though it seems to be a part of the physiological pharyngeal flora in healthy domestic cats (8), *M. felis* also acts as an opportunistic pathogen causing various diseases in different hosts (3, 7, 9).

In this study *M. felis* strains isolated from domestic cats, wild *Felinae* and horses were characterized using genomic and proteomic approaches. For genomic characterization, whole genome sequences generated in this study were subjected to core genome multi-locus sequence typing (cgMLST) followed by determining the phylogenetic relationship between these strains. For proteomic characterization, protein mass fingerprints (spectra) were generated from cultures by matrix assisted laser desorption ionization – time of flight (MALDI-ToF) mass spectrometry and compared to determine the degree of spectral concordance between the investigated *M. felis* strains.

The aim of this thesis was to determine the relatedness of *M. felis* strains predominantly isolated from domestic cats but also from wild *Felinae* and horses. For this purpose, genomic and proteomic typing methods were employed to investigate the epidemiology of *M. felis* infection and to gain insights into the population structure of this fascinating, primarily feline pathogen.

2. Literature

2.1. General characteristics of mycoplasmas

Mycoplasmas are bacteria classified within the class *Mollicutes*, distinguished from other bacteria by their absence of a cell wall. Because of their small cell and genome size, members of this class are considered the smallest prokaryotes with the ability to self-replicate (10) and are believed to represent bacteria with a minimal set of genes required for independent life (11). Another characteristic defining mycoplasmas is their low genomic G+C content, the presence of cholesterol in the cell membrane and the codon usage of UGA for tryptophan (4). Various mycoplasmas demonstrate remarkable genetic plasticity and rapid evolution. This is primarily driven by spontaneous mutations and recombination within a given species, as well as horizontal gene transfer (HGT) occurring both between and within species. These mechanisms enable them to quickly adapt to changing environments, enhancing their survival and potentially increasing their virulence (12–14). Mycoplasmas have restricted abilities for both anabolism and metabolism due to their reduced genome. As a result, they adopt a strict parasitic lifestyle, mainly depending on nutrients from their host (4).

To date, more than 200 different mollicutes species have been detected in or isolated from humans, animals and plants. Most of these mollicutes are members of genus *Mycoplasma*. Mycoplasmas found in animals typically show a strict host and tissue specificity. Their habitats include the respiratory tract, the urogenital tract, eyes, joints, the udder and serous membranes of body cavities (4). While certain species are well recognized as primary or opportunistic pathogens causing diseases or contributing to disease development, others are harmless commensals coexisting as part of the physiological flora (15). Pathogenic mycoplasmas are not considered highly virulent, mainly causing slowly progressive chronic infections rather than severe diseases. In several mycoplasmas, the mechanisms of pathogenicity and virulence remain largely unknown although determinants of pathogenicity like adhesins, capsular polysaccharides, and variably expressed surface lipoproteins have been identified in some *Mycoplasma* species, particularly in those that cause well-recognized diseases in livestock and poultry. The transmission of mycoplasmas occurs primarily through direct contact between animals and is mostly airborne. However, other routes of infection such as through blood, milk or genital contact are also known. Vertical transmission has also been documented (16).

2.2. General characteristics of *Mycoplasma felis*

In 1967, *M. felis* was validly described as new species within genus *Mycoplasma* by Cole et al. In their study, five strains of unknown mycoplasmas, one isolated from the eye of a cat displaying severe conjunctivitis and four strains recovered from the saliva of healthy cats were analyzed using morphological, biochemical and antigenic characterization methods. Based on biochemical and antigenic properties two distinct species were recognizable and the name *M. felis* was proposed for the strain isolated from the conjunctivitis case (type strain CO) and for two isolates from the saliva, whereas the name *M. gateae* was proposed for the two remaining oral strains (17).

Colonies of *M. felis* present themselves with a classic fried egg morphology, but the opacity of colonies may differ significantly. The cell morphology of *M. felis* varies from spherical to coccoid. Its biochemical property to acidify the culture medium by fermentation of glucose through glycolysis distinguishes *M. felis* from *M. gateae*, which alkalinizes the culture medium by hydrolysis of arginine (17).

Based on 16S rRNA gene phylogeny and phenotypic characteristics, *M. felis* is a species within genus *Mycoplasma*, which belongs to the family *Mycoplasmataceae* and the order *Mycoplasmatales*. Alongside four other orders, *Mycoplasmatales* is a member of the class *Mollicutes*, which is the only class of the phylum *Mycoplasmata* (18). Within genus *Mycoplasma* *M. felis* is phylogenetically positioned in the *M. synoviae* cluster of the Hominis group. Its closest relative is *M. mustelae*, a mink mycoplasma, exhibiting a 16S rRNA gene sequence similarity of 96.61% to *M. felis* (1).

The complete genome sequence of *M. felis* type strain CO^T was not deposited at the NCBI (National Center for Biotechnology Information) genome database until 2024. The circular chromosome of *M. felis* CO^T has a size of 919,617 bp and a genomic G+C content of 24.5%. Overall, the genome contains a total of 799 genes, 753 predicted protein coding DNA sequences (CDS), three sets of complete rRNAs (16S, 23S, 5S), and 30 tRNAs.

To date, 23 fully sequenced genomes of *M. felis* have been made publicly available including genomes of one equine and 22 feline *M. felis* strains. The first-ever fully sequenced *M. felis* strain, designated Myco-2, was isolated in 2010 in Japan from the trachea of a wild horse (*Equus ferus*) displaying signs of respiratory disease. The remaining 22 *M. felis* strains with finished genomes were predominantly isolated from the respiratory tract of diseased domestic cats in Australia and Canada. In addition, seven draft genomes of feline *M. felis* strains have

been deposited at the NCBI genome database (www.ncbi.nlm.nih.gov/datasets/genome/?taxon=33923) (19).

The latest genomic research on *M. felis* indicated unique gene acquisitions through horizontal gene transfer (HGT) between different *Mycoplasma* species that share feline as well as canine hosts and *M. felis*. Further key findings of this study included the presence of a mycoplasma bacteriophage in one of the feline *M. felis* strains and the acquisition of the virulence-associated glycerol-3-phosphatase (GlpO) gene in all feline strains, which was absent in the only genome-sequenced equine *M. felis* strain, Myco-2. Furthermore, gain and loss of additional proteins pointed to potential differences in bacterial growth and replication strategies between feline and equine *M. felis* strains. Therefore, it was suggested that *M. felis* infections may vary between hosts, and further research is needed to evaluate host-specific infection dynamics. Additionally, rapid evolution and host adaptation of *M. felis* was observed, as genomic changes occurred within two months in one feline host (19).

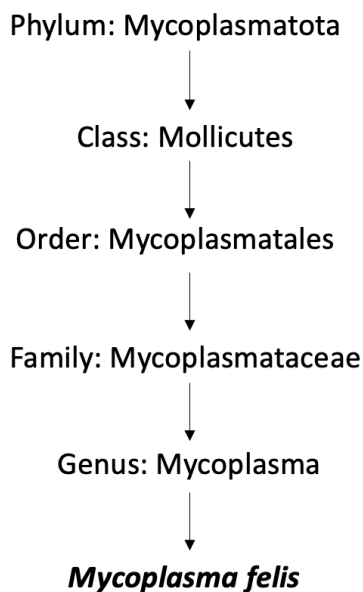


Figure 1: Taxonomic classification of *Mycoplasma felis*

2.3. *Mycoplasma felis* and associated diseases

M. felis is a common inhabitant of the oropharynx and the upper respiratory tract of *Felinae* (small cat subfamily of *Felidae*) including domestic cats, and may less frequently be present in the respiratory tract of horses (1). This opportunistic pathogen is also frequently associated with conjunctivitis and respiratory disease in cats and occasionally with pneumonia and pleurisy in horses (4).

2.3.1. *Mycoplasma felis*-associated diseases in domestic cats

M. felis can cause various diseases in domestic cats including conjunctivitis (6) respiratory tract diseases (2), and arthritis (7).

M. felis is commonly associated with co-infection with viral (e.g., feline calicivirus) and bacterial (e.g., *Bordetella bronchiseptica*, *Chlamydia felis*) pathogens that cause chronic and acute upper respiratory tract disease (URTD) and is less commonly implicated in lower respiratory tract disease (LRTD) of domestic cats (3, 8, 20–24). In a case-control study, *M. felis* was not detected in the group of healthy cats, but in a significant number of cats with signs of URTD. Moreover, healthy in-contact cats living in the same household also tested positive for *M. felis*, which may act as carriers and possible source of reinfection (2).

Studies have also shown that approximately 22 % of cats with lower respiratory disease may have a concurrent *M. felis* infection while the pathogen was shown to be absent in the lungs of healthy cats. In addition, an experimental challenge with *M. felis* in kittens induced pneumonia highlighting the role of *M. felis* as primary pathogen in the lower respiratory tract. Furthermore, naturally occurring *M. felis* infections are increasingly reported in domestic cats associated with suppurative bronchitis, bronchiolitis and / or pneumonia. Predisposing factors can play an important role in the development of clinical signs of respiratory mycoplasma infection. These include triggers such as young age, immunosuppression, exposure to cigarette smoke, syndemic viral/bacterial infections or underlying diseases like periodontal disorders (3).

Additionally, *M. felis* has been identified as the sole pathogen causing mono-arthritis in two domestic cats. In these two cases, clinical signs included non-weight-bearing lameness, elevated body temperature and decreased general behavior. As no injuries were found in the area of the affected joints, it was assumed that the pathogen had entered through distant bite wounds and had spread to the joints via the hematogenous route (7).

M. felis is also considered a significant pathogen in feline conjunctivitis. The prevalence rate of the *M. felis* in ocular swabs collected from cats with conjunctivitis was 9.6% or 25 % which is significantly higher than the rates present in the eye of healthy cats (2.3 % or 0 %, respectively) (25). Experimental infections of cats have produced conjunctivitis in kittens when a high infection dose was used (6).

2.3.2. *Mycoplasma felis* in wild *Felinae*

The occurrence and role of *M. felis* in wild *Felinae* has not been investigated so far. However, one case report describing the isolation of *M. felis* infection in a 3-week-old serval (*Serval felis*) suggests that *M. felis* also occurs in wild *Felinae*. In the case described, a transtracheal lavage was performed on a serval cub displaying severe respiratory signs and the sample submitted for microbiological examination. *M. felis* was the only bacterial pathogen detected in significant quantities. The results of the cytological blood test also confirmed a severe bacterial infection. In addition, the medical history indicated an immunodeficiency with a lack of maternal antibodies after the cub was bottle-fed and did not receive colostrum. It is important to note that its two litter mates did not show any symptoms, but an asymptomatic process cannot be ruled out (9).

2.3.3. *Mycoplasma felis* in horses

In earlier studies on horses, *M. felis* has been identified as the causative agent of diseases of the respiratory tract and the serous membranes of the thoracic cavity, such as pneumonia, pleurisy and pericarditis (10, 11, 21). In a previous study, 8% of horses suffering from respiratory disease were shown to be infected with *M. felis* (26) and it was suspected that *M. felis* may act as a primary pathogen of LRTD in horses (27). Clinical signs of *M. felis* infection included depression, coughing, nasal discharge and pyrexia (26, 27). In 1991, *M. felis* was identified as cause of pleurisy in two Canadian show jumper horses (4). Additionally, high antibody titers against *M. felis* were found by serologically testing horses with a recent history of pleurisy (28). Furthermore, *M. felis* was detected in pericardial effusions that were the product of pericarditis (4, 5). Today, *M. felis*-associated diseases in horses are rarely diagnosed, since clinical samples from the equine respiratory tract and thoracic cavity are not routinely tested for mycoplasma (5).

2.4. Laboratory diagnosis of *Mycoplasma felis*

Certain clinical specimens are appropriate for the laboratory diagnosis of *M. felis* infections in cats and horses. In cases of LRTD, bronchoalveolar lavage samples are well-suited (3) whereas in URTD oropharyngeal swabs or tracheal lavage samples should be taken (2). From cats displaying conjunctivitis, conjunctival swabs should be collected (29). In arthritis cases either joint fluid aspirates (30) or swabs taken from the joint cavity after synovectomy are suitable specimens for the laboratory diagnosis of *M. felis* (7). To diagnose pleuritis or pericarditis caused by *M. felis*, pleural fluid should be punctured and examined microbiologically (5).

Laboratory diagnosis of *M. felis* infection is commonly achieved by utilizing conventional cultivation procedures. Several culture media suitable for the propagation of mycoplasmas like SP4 (31), Eaton's (32), Hayflick's (33) and Friis medium (34) can be employed. On agar plates, *M. felis* produces typically fried egg-shaped colonies after incubation at 37°C and 5-10% CO₂-atmosphere for 2-5 days. Since *M. felis* is a glucose fermenter, a color change of the broth medium based on the release of organic acids during glycolytic processes is visible (17).

Mycoplasma cultures are traditionally identified at the species level by genetic methods such as 16S rRNA gene, 16S-23S intergenic spacer or *rpoB* gene sequencing. More recently, MALDI-TOF mass spectrometry has been shown to be a rapid and accurate method for the identification and differentiation of mycoplasmas isolated from animals including *M. felis* (15).

Several conventional and real-time polymerase chain reaction (PCR) systems have been developed for the species-specific detection of *M. felis* in clinical specimens and their use provides the advantage that no cultural pre-enrichment is required for PCR detection (35–37).

2.5. Typing methods for mycoplasmas

Molecular typing methods are currently preferred for the characterization of mycoplasma strains in order to determine epidemiologic characteristics of mycoplasma infections such as transmission modes and infection routes.

MALDI-ToF mass spectrometry is a suitable molecular tool for the phenotypic characterization of mycoplasmas. MALDI-ToF phenotyping is based on the spectrometric analysis of bacterial ribosomal proteins and the comparison of the resulting protein mass fingerprints (38–41).

For the genotypic characterization of mycoplasmas, multi-locus sequence typing (MLST) has been employed successfully for several years. This typing method usually analyzes five to seven housekeeping genes for allelic differences. In contrast to phenotypic typing methods, changes are detected at the DNA level by investigating sequence data. A major advantage of MLST is that no cultured microorganisms or high-quality genomic DNA are required for typing. However, despite these advantages, there is still no MLST scheme available for typing *M. felis* (42–45).

In recent years, core genome MLST (cgMLST) schemes have been developed for certain mycoplasma species such as *M. hyorhinis*, *M. gallisepticum*, and *M. bovis*, but none is available for *M. felis*. The cgMLST is an extension of the classical MLST characterized by a high-resolution capacity since the cgMLST analyzes hundreds of genes of the core genome instead of 7 housekeeping genes that are examined by MLST. However, a prerequisite for cgMLST is the availability of whole genome sequence data of the mycoplasma strains to be analyzed (46–48).

3. Material and Methods

3.1. *Mycoplasma felis* strains

A total of 49 *Mycoplasma felis* isolates and the type strain CO^T were included in the study. All isolates were obtained from clinical samples over a period of more than 20 years and cryopreserved at -80°C. Of the 49 isolates, 41 were recovered from domestic cats (Table 1), while six isolates originated from wild *Felinae* and two from horses (Table 2). Of seven domestic cat isolates, two cryopreserved colony variant cultures, namely of opaque and lucent colony types, were included in the study.

Table 1: *M. felis* isolates from domestic cats including the type strain analyzed in the presented study

Strain designation	Year of isolation	Sample type
CO ^T (type strain)	1967	Conjunctiva
1710	2000	Joint
1206	2003	Joint
2666	2003	Thorax
880	2006	Thorax
2713	2009	Serosa
431	2011	Joint
4312	2011	Placenta
1392	2012	Joint
2193	2015	BAL
544	2015	Thorax
837d*/h**	2015	BAL
734d*/h**	2016	BAL
756d*	2016	BAL

1370d*/h**	2016	BAL
456	2017	Aspirate (lung)
2210h**	2018	BAL
514	2018	BAL
2241	2018	Organ pool
342	2019	BAL
4170d*/h**	2019	BAL
1945Ad*/Bh**	2019	BAL
2063	2019	BAL
2510d*/h**	2019	Nose
1127d*	2019	BAL
1578	2019	Lung
1038h**	2019	BAL
1631	2019	BAL
1291d*/h**	2020	BAL
3521	2020	BAL
2602	2020	BAL
33	2021	Lung
1851	2021	BAL
1399	2021	BAL
233	2021	BAL
597	2021	BAL
3661	2021	Synovia

1610	2023	Pharynx
735	2023	Nose
335	2023	Lung
336	2023	Lung
713c	2023	Cornea

*opaque (d stands for 'dunkel' in German) colony type

**lucent (h stands for 'hell' in German) colony type

Table 2: *M. felis* isolates from wild *Felinae* and horses analyzed in the study

Strain designation	Year of isolation	Sample type	Host species
1448	2002	Nose	Horse
1099	2002	BAL	Horse
713	2010	Lung	European wildcat
4986	2016	Lung	European wildcat
853	2019	Trachea	Cheetah
AC1062	2020	Trachea	European wildcat
Z43A	2020	Lung	European wildcat
Z885	2021	Trachea	Lynx

3.2. Cultivation of the *Mycoplasma felis*

First, approximately 50 µl of the cryopreserved cultures were thawed and streaked onto SP4 agar plates (Institute of Microbiology, Vetmeduni, Austria) using an inoculation loop. Agar plates were then incubated at 37°C under 5% CO₂ atmosphere for 2-4 days. After this incubation period the agar plates were checked for colony formation using a stereomicroscope. As soon as colonies were produced on agar plates, a single colony was picked with a Pasteur pipette, transferred into 3 ml SP4 liquid medium (Institute of Microbiology, Vetmeduni, Austria) and incubated at 37°C and ambient air for 2-4 day until a color change of the medium from red to orange or yellow was observed.

The cultures were then aliquoted evenly into four Eppendorf tubes. Two tubes were immediately frozen at -80°C as backup cultures, while the other two aliquots were centrifuged at 20.000 x g for seven minutes, and the supernatant subsequently discarded. The pellets obtained were then stored at -20°C until further analysis, i.e., MALDI-TOF mass spectrometry and Illumina sequencing.

3.3. Genomic characterization of *Mycoplasma felis*

3.3.1. DNA extraction

The MagAttract® HMW (High Molecular Weight) DNA Extraction Kit (Qiagen N.V., Netherlands) was used to extract DNA from cultured strains.

The first step of DNA extraction encompassed thawing the frozen pellet obtained from cultures and subsequently adding 180 µl ATL Lysis Buffer to dissolve the pellet.

Then, 20 µl Proteinase K solution and 4 µl RNase were mixed with the suspension. After adding 150 µl of AL buffer, the mixture was incubated at 56°C for 30 minutes in a thermal mixer (Eppendorf AG, Hamburg, Germany) shaking at 900 rpm.

After cooling down for two minutes at room temperature, 15 µl MagAttract-Suspension G (containing magnetic beads) and 280 µl of MB Binding Buffer were added. Afterwards, the sample was vortexed and incubated again in the thermomixer (1400rpm) for three minutes at room temperature. The tube was then transferred into a magnetic rack (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and remained there until the beads with bound DNA were separated from the supernatant. The liquid was then carefully removed until only the beads remained. Then, the beads were washed by adding 700 µl MW1 Wash Buffer and

shaking in a thermal mixer for one minute at 14000 rpm, followed by removal of the residual washing solution. This step was then repeated once using the MW1 Wash Buffer and subsequently repeated twice using the PE Wash Buffer. When the supernatant was discarded after the last cleaning step, the tubes remained in the MagAttract magnetic rack and 700 µl distilled water were added without releasing the water directly onto the beads. The sample was incubated for one minute and the liquid was discarded. This step was repeated. Afterwards, 80 µl of AE Elution Buffer were added and the tubes were placed into the thermomixer (1400 rpm) once again for three minutes at room temperature. After placing the tube in the magnetic rack until the beads were separated, the DNA extract was carefully transferred into a new tube. Subsequently, the concentration of the DNA was measured by using the Qubit 4 Fluorometer (Thermo Fisher Scientific) and the tubes were then stored at -20°C until further usage.

3.3.2. Library preparation for Illumina sequencing

The "Illumina DNA Prep Reference Guide" and the necessary reagents (Illumina, Inc., USA) were used to prepare the libraries for Illumina sequencing. The first step was to thaw the tubes, which contained the extracted DNA. Afterwards, 30 µl of DNA extract (containing a total amount 100-500 ng of DNA) of each sample was transferred into a new sterile tube. For the so called 'tagmentation' of genomic DNA, bead-bound transposomes were used to fragment the DNA and add adapter sequences. First, the Tagmentation Master Mix was prepared by mixing 11 µl of Bead-Linked Transposomes (BLT, Illumina, San Diego, California, USA)) with 11 µl of Tagment Buffer 1 (TB1, Illumina). Next, to 20 µl of Tagmentation Master Mix were added to the sample and carefully mixed using a pipette. The sample was then transferred into PCR tubes (Eppendorf SE) and placed into the PCR thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) to start the TAG program. For this, the thermocycler was set to preheat the lid to 100°C, the reaction volume was adjusted to 50 µl, and the reaction mix was incubated at 55°C for 15 minutes. After tagmentation, the sample was cleaned up by adding 10 µl of Tagment Stop Buffer (TSB, Illumina). The tube was then transferred into the thermal cycler again to start the PTC program. For this program the same settings as in the TAG program were used, only the temperature was reduced to 37°C. After finishing this process, the sample was transferred into a new tube and placed in a magnetic racket for three minutes. The supernatant was then removed until only the tagmentation beads remained. For the washing procedure, the tube was placed in a non-magnetic rack and the beads were resuspended using 100 µl Tagment Wash Buffer (TWB, Illumina). To separate the washing

buffer from the beads, the tubes were replaced in a magnetic rack until the liquid was clear and the supernatant was discarded again. This washing step was repeated twice, with the liquid not being removed during the second repetition.

Next, the amplification of the tagmented DNA was performed. For this purpose, a PCR program was used to add the Index-1-Adapter (i7) and the Index-2-Adapter (i5) to the sequences. First, the PCR Master Mix was prepared by mixing 22 μ l of Enhanced PCR Mix (EPM, Illumina) and 22 μ l of nuclease-free water per sample. Before starting the amplification process of the tagmented DNA, the supernatant was discarded and the sample was placed into a non-magnetic rack. Afterward, 40 μ l of the PCR Master Mix was added to each sample, ensuring thorough mixing, so that the beads were fully suspended. For sample barcoding, 10 μ l of index adapter (IDT for Illumina RNA/DNA UD Indexes, Illumina) were added, mixed and the sample transferred into a PCR tube. Next, the amplification process was initiated by placing the sample into the thermal cycler and starting the BLT program with the following cycling conditions: three minutes at 68°C, three minutes at 98°C, five cycles each at 98°C for 45 seconds, at 62°C for 30 seconds and at 68°C for two minutes, and a final extension step at 68°C for one minute.

In the next step the amplified libraries were subjected to a cleaning process by using double-sided beads. For this, the samples were returned to the magnetic rack again. Once the supernatant was clear, it was transferred to a new tube. Afterwards, 40 μ l nuclease-free water and 45 μ l of Illumina Purification Beads (IPB, Illumina) were added. The samples were then thoroughly mixed with a pipette and incubated at room temperature for five minutes, followed by another incubation step of five minutes on the magnetic rack. During this time new tubes were prepared by adding 15 μ l IPB. After incubation the suspension was transferred into the prepared tube containing IPB, mixed and allowed to incubate for an additional five minutes before it was placed again into the magnetic rack until the supernatant was clear. The supernatant was then carefully removed without touching the beads. Next, the beads were washed by adding 200 μ l 80% EtOH from the opposite side of the wall to avoid dissolving the beads. After an incubation period of 30 seconds, the EtOH was removed. Afterwards, the tube remained 5 more minutes in the magnetic rack to air dry and was then transferred into a non-magnetic rack. Subsequently, 32 μ l of Resuspension Buffer (RSB, Illumina) was added. After two minutes the tube was once again placed into the magnetic rack until the supernatant was clear. Finally, the library was transferred into a new tube and stored at -20°C until all samples were prepared for Illumina sequencing.

3.3.3. Illumina whole genome sequencing

Protocol A of the MiniSeq System (Illumina) was used to prepare the *M. felis* samples for genome sequencing. As a first step 5 µl of each sequencing library was transferred into one Eppendorf tube to create a pool sample. The DNA content of the library pool was then measured using the Qubit 4 Fluorometer (Thermo Fisher Scientific). Subsequently, the library pool was diluted to a concentration of 2 nmol for denaturation in a following step and then further diluted to a loading concentration of 1,3 pmol.

To dilute the library pool to a starting concentration of 2 nmol the following formula was used, and the Qubit measurement was inserted (ng/µl) to calculate the molarity of the pool:

$$\text{molarity} = \frac{(\text{ng}/\mu\text{l})}{660 \frac{\text{g}}{\text{mol}} \times 600 \text{ bp}}$$

The total volume of 50 µl, which includes the correct volume of the library pool and Resuspension Buffer (RSB, Illumina, Inc., USA), was calculated using the following formula:

$$x \mu\text{l} = \frac{2 \text{ nmol} \times 50 \mu\text{l}}{y \text{ nM}/\mu\text{l}}$$

x ... volume of library pool (µl)

y ... calculated molarity

To calculate the volume of RSB, the calculated volume of the library pool (x) is subtracted from the total volume of 50 µl.

The calculated library pool volume was then transferred into a new tube along with the correct amount of resuspension buffer (RSB) resulting in a total volume of 50 µl. The 2 nM pool was then diluted to 1nM by adding 50 µl of RSB. To denature the library 5 µl of the 1nM diluted library pool was transferred into a new tube and 5 µl of 0.1 N NaOH was added, followed by a strict five-minute incubation period at room temperature. To stop the denaturing process, 5 µl of RSB was added to the tube. Afterward, 985 µl hybridization buffer (HT1, Illumina) was added to the tube to dilute the suspension to 5 pmol. Finally, 130 µl of this dilution was transferred into a new tube and 370 µl HT1 was added to achieve a loading concentration of 1,3 pmol.

Subsequently, the MiniSeq platform (Illumina) was loaded with a Mid output flowcell (Illumina) together with a washing cartridge (Illumina). The diluted and denatured library pool was then transferred onto the flow cell and the sequencing program was started. After 17 hours the sequencing run was completed and the raw sequence data, separated by indices, were

uploaded to the Geneious Prime software platform (Biomatters, Auckland, New Zealand). Following quality filtering and trimming of raw reads, draft genomes were assembled by SPAdes, resulting in the generation of contigs (sets of overlapping DNA fragments).

3.3.4. Core genome multilocus sequence typing

The Ridom SeqSphere+ version 7.0 software (Ridom GmbH®, Münster, Germany) was used to establish a cgMLST scheme for *M. felis* as described previously for other *Mycoplasma* species (46, 47, 49). The closed reference genome of equine *M. felis* strain Myco-2 was downloaded from the NCBI genome database and uploaded to the Ridom SeqSphere platform along with four query genomes including the draft genomes of type strain CO^T and strains 735, 837, and 4312 sequenced in this study. At first, target genes in the reference genome appropriate for cgMLST were defined with the assistance of the target definer tool that excluded unfit genes using different filter setups. Afterward, the four query genomes underwent pairwise comparison against the target gene set of the reference genome to identify commonly present and suitable targets presenting sequence identities of > 90% and full overlap. After the development of the typing scheme, all draft genomes generated during this thesis were analyzed to define individual allelic profiles. Subsequently, a phylogenetic tree was constructed from the resulting allelic profiles employing the neighbor-joining algorithm to illustrate the phylogenetic relationships between the *M. felis* strains studied.

3.4. Proteomic characterization

3.4.1. MALDI-TOF mass spectrometry

For proteomic characterization MALDI-TOF mass spectrometry was used to generate and compare individual mass fingerprints of *M. felis* strains investigated in this study.

First, the frozen tubes containing pellets obtained from cultures of *M. felis* strains (see 3.2) were thawed and 100 µl of H₂O (HPLC-Grad, Sigma-Aldrich, Austria) was added to each tube to resuspend the pellet. The tubes were then centrifuged at 20.000 x g for five minutes. After removal of the supernatant, the remaining pellet was resuspended with 15 µl of 70% formic acid (Sigma-Aldrich, Austria) and 15 µl of 100% acetonitrile (Sigma-Aldrich, Austria) for protein extraction. The tubes were then centrifuged at 20.000 x g for two minutes. Afterward, 1 µl of each obtained protein extract was spotted onto the MALDI-ToF target plate. After air-drying

each spot was overlaid with 1µl α-cyano-4-hydroxycinnamic acid matrix solution (Bruker Daltonics, Germany). After an additional air-drying period the target plate was introduced into the MALDI-TOF MS Microflex LT Biotyper (Bruker Daltonics, Germany), and the corresponding computer program FlexControl 3.4 (Bruker Daltonics, Germany) was started. Each inoculated spot on the target plate was manually shot by a nitrogen laser using the linear, positive ion mode, which generated a specific spectrum for each individual *M. felis* strain. Afterwards, the individual mass fingerprints underwent a quality control via the FlexAnalysis 3.4 Software (Bruker Daltonics, Germany). In a final step, the generated spectra were transferred to MBT Compass Explorer Software (Bruker Daltonics, Germany) and a dendrogram based on arbitrary distance matrix was constructed using the correlation distance measure with the average linkage algorithm.

4. Results

4.1. Genomic characterization

4.1.1. Development of a *M. felis* cgMLST scheme

From the finished genome of equine *M. felis* strain Myco-2, a reference task template was generated that contained 568 genes after filtering. By using four query genomes (CO^T, 735, 837, 4312), a cgMLST scheme with 454 target genes was finally defined, covering approximately 45% of the *M. felis* Myco-2 genome. For evaluation of the developed cgMLST scheme, all draft genomes of *M. felis* strains generated during this study were uploaded into Ridom SeqSphere+ and analyzed according to the 454 cgMLST scheme. At least 97% cgMLST target genes were found in the genomes used for evaluation confirming that unfit genes were successfully excluded from the typing scheme. The average number of alleles for 454 target genes was 36 ranging from eight to 52 alleles indicating a high differentiation potential and resolution capacity of the established cgMLST scheme for *M. felis* strains.

4.1.2. Typing of *M. felis* strains using cgMLST

A phylogenetic tree constructed from allelic profiles resulting from cgMLST illustrates that the examined *M. felis* strains possess a high level of diversity. In the tree the strains were roughly separated into two clusters: cluster A comprises 36 strains (genomes) isolated from domestic cats, wild *Felinae* and horses, whereas cluster B contains 22 *M. felis* strains that were solely isolated from domestic cats. In cluster A, wild *Felinae* strains Z885 (lynx), 713 (wildcat), and 4986 (wildcat) grouped together forming a subcluster (A1). In addition, a further subcluster (A2) was observed within cluster A comprising the three equine *M. felis* strains (genomes) analyzed. Most of the isolates which were separated into two different colony morphotypes (opaque [d], lucent [h]) and sequenced separately, appeared to be closely related, except for the colony opacity variants 2510d and 2510h that were shown to be more distantly related. In the phylogenetic tree, no link between the genome-wide relatedness and the geographic and temporal origin of *M. felis* strains were recognizable.

Tree scale: 0.1

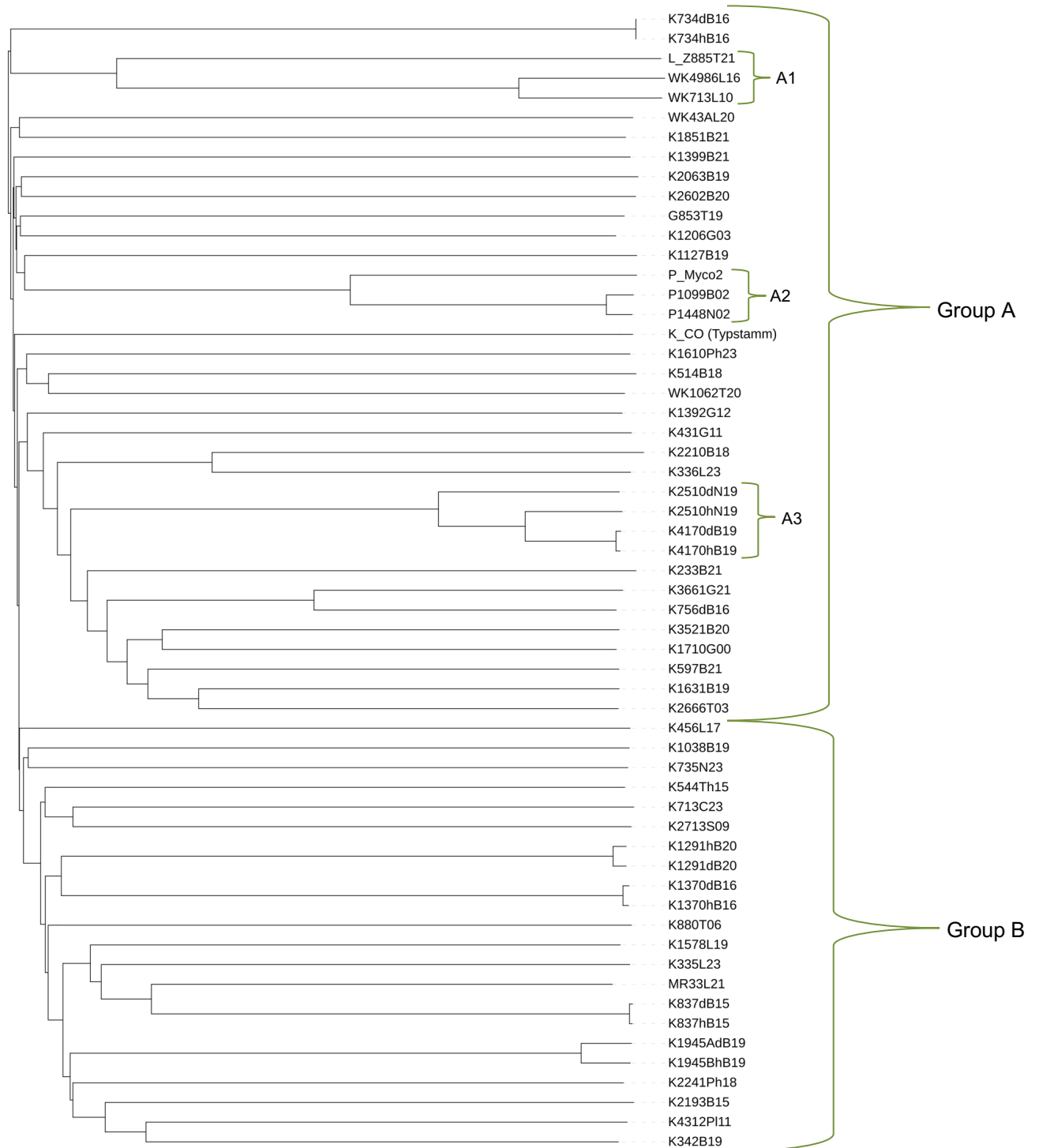


Figure 2: Phylogenetic tree generated from allelic profiles resulting from cgMLST of 58 *M. felis* strains and colony variants

4.2. Phenotypic characterization

4.2.1. Colony opacity variants

M. felis colonies typically exhibited the characteristic fried-egg morphology with smooth margins. Two distinct morphotypes were identified: opaque [d] and lucent [h] colonies. In general, lucent colonies appeared visually larger than their opaque counterparts. Upon examination of the *M. felis* isolates, three general patterns of colony morphology were observed: some isolates consistently formed opaque [d] colonies with a dense appearance, others exclusively displayed lucent [h] morphotypes. The third group exhibited both morphologies simultaneously. Notably, two isolates initially classified as opaque displayed only lucent colony morphotypes upon further examination. Since a lucent isolate had already been selected from each, these isolates were excluded from further analysis.

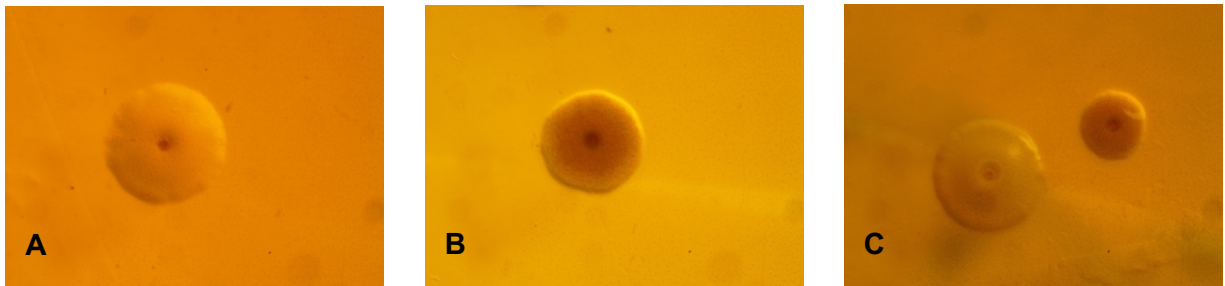


Figure 3: Representative colony morphotypes of strains 1291h (A), 2510d (B), and 1291d (C) grown on SP4 agar plates and imaged after 48 hours of incubation at 40× magnification.

4.2.2. MALDI-TOF mass spectrometry

A dendrogram was constructed based on individual mass spectra generated from a total of 57 *M. felis* strains and colony opacity variants using MALDI-TOF mass spectrometry. In the dendrogram, the analyzed strains were clearly separated into two clusters: cluster C comprises 14 strains, while cluster D consists of 45 strains. Cluster D is predominantly composed of strains from domestic cats except strains 1448 (horse) and 853 (cheetah). Overall, the dendrogram does not show any correlation between cluster assignment and strain attributes such as host species, sample type, time of isolation, and phylogenetic relatedness.

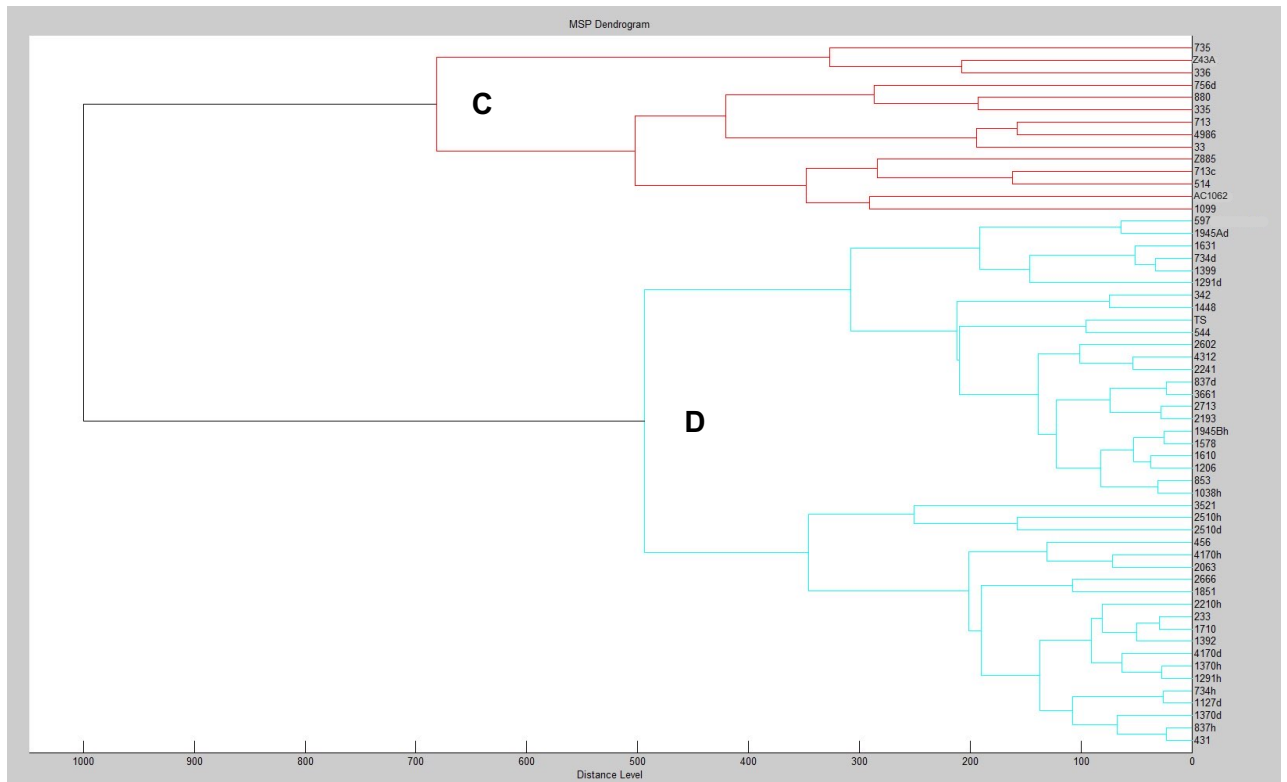


Figure 4: Dendrogram derived from similarity matrices based on MALDI-ToF mass spectra of 57 *M. felis* strains and colony opacity variants

5. Discussion

Mycoplasma felis is a globally distributed opportunistic pathogen predominantly affecting domestic cats, and occasionally wild felids and horses, where it is associated with a spectrum of clinical manifestations ranging from conjunctivitis and respiratory tract infections to pleuritis and arthritis (1–7). Comprehensive genomic data and a precise understanding of phylogenetic relationships and the epidemiology are critical for driving research progress. Therefore, the aim of this thesis was to characterize 49 *M. felis* isolates from domestic cats, wild *Felinae*, and horses using cgMLST and MALDI-TOF mass spectrometry, to investigate their phylogenetic relationships, epidemiology, and population structure.

MALDI-TOF MS is frequently used for proteomic characterization, as it has proven to be a reliable method for differentiating *Mycoplasma* isolates, even within a given species, based on characteristics such as year of isolation or host origin, allowing for the clustering of strains with similar spectral profiles (15, 39, 40). The *M. felis* isolates analyzed in the present study were grouped into two distinct clusters (Group C and Group D; Figure 5) based on the dendrogram generated via MALDI-TOF mass spectrometry. However, the clustering observed did not reflect any detectable epidemiological pattern, as the isolates within each group did not share consistent attributes such as date of isolation, geographic origin, host species, or sample type.

MALDI-TOF MS appeared to be an inadequate method for supporting genetic relationships within *M. felis* isolates, as evidenced by the discrepancies between the dendrogram constructed from spectral data and the phylogenetic tree. The diverging performance might also be a consequence of insufficient standardization of the culturing process of isolates for MALDI-ToF MS as previous studies indicate that the quality of the mass spectra generated depends strongly on the growth phase of the cultures analyzed. Further research should focus on the determination of strain-specific growth curves to identify the optimal growth phase of *M. felis* cultures that can be used to obtain high-quality MALDI-TOF mass spectra (15, 38, 50).

In this study, a newly established cgMLST scheme for *M. felis* was presented, which was shown to provide a high differentiation potential and resolution capacity to distinguish *M. felis* strains. These findings are consistent with previous studies demonstrating that cgMLST offers superior discriminatory power compared to conventional MLST methods, and is therefore better suited for elucidating genetic relationships (46, 49).

The phylogenetic tree constructed using the cgMLST scheme revealed genetic relatedness among *Mycoplasma felis* isolates with shared epidemiological backgrounds, despite the

overall high genomic diversity and the absence of clear genome-wide clustering. A group of wild feline isolates (4986, 730, Z885) formed a distinct cluster (A1; Figure 2), while the remaining wild feline samples were dispersed throughout group A, suggesting that no specific genomic variants delineate a wild feline-associated subtype. Equine isolates formed a separate cluster (A2; Figure 2), indicating potential host-specific phylogenetic clustering. However, this result should be interpreted with caution as it is based on only three strains of *M. felis* from horses, two of which came from the same clinic and were isolated in the same year, suggesting nosocomial transmission. Both Austrian isolates were closely related to the equine reference strain Myco-2 but showed no further geographic or temporal links beyond the host species, supporting the hypothesis of host-specific clustering. Given the limited dataset, further studies with a broader set of equine isolates are needed to validate this observation. In contrast to MALDI-ToF MS, the cgMLST-based phylogeny revealed close genetic relationships between opaque [d] and translucent [h] colony morphotypes derived from the same isolates, except for the morphotype pair 2510d/2510h, which exhibited greater genetic distance. This discrepancy suggests that phenotypic variation in colony morphology (opacity) is related to differential expression of proteins whose encoding genes are apparently not part of the core genome and therefore cannot be differentiated using cgMLST.

The cgMLST analysis revealed pronounced genomic diversity among *M. felis* isolates, characterized by substantial allelic distances. Such heterogeneity is consistent with findings from previous cgMLST studies on other *Mycoplasma* species, including *M. gallisepticum*, *M. hyorhinis*, *M. hyosynoviae* and *M. anserisalpingtonis* (46, 47, 51, 52). To gain deeper insights into the genomic structure of *M. felis*, future studies should focus on in-depth comparative analyses that may reveal genomic features linked to virulence, host adaptation, cytotoxicity, and antimicrobial resistance, ultimately providing a foundation for improved therapeutic and preventive strategies (19).

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