POLYPHASIC CHARACTERISATION OF UNCLASSIFIED MYCOPLASMA ISOLATES RECOVERED FROM VARIOUS HOSTS
Index

1 Introduction ................................................................................................................................. 1

1.1 Mycoplasmas ............................................................................................................................. 1

1.1.1 Taxonomy .............................................................................................................................. 1

1.1.2 Ecology and habitat .............................................................................................................. 1

1.1.3 Morphology .......................................................................................................................... 2

1.1.4 Physiology and metabolism ............................................................................................... 2

1.1.5 In vitro cultivation ................................................................................................................. 3

1.1.6 Genome composition and phylogeny ................................................................................. 4

1.2 Aim of the project ..................................................................................................................... 5

2 Material and method .................................................................................................................. 6

2.1 Mycoplasma strains .................................................................................................................. 6

2.1.1 Mycoplasma isolates .......................................................................................................... 6

2.1.2 Control strains .................................................................................................................... 8

2.2 Cultivation of mycoplasmas .................................................................................................... 9

2.2.1 SP4 medium ......................................................................................................................... 9

2.2.2 PPLO base medium ............................................................................................................ 10

2.3 Cell and colony morphology ................................................................................................. 11

2.3.1 Colony morphology ............................................................................................................ 11

2.3.2 Filtration ............................................................................................................................. 12

2.4 Physiology and metabolism ................................................................................................. 12

2.4.1 Optimum growth temperature and oxygen requirement ................................................ 12

2.4.2 Film and spots .................................................................................................................... 12

2.4.3 Digitonin sensitivity .......................................................................................................... 13

2.4.4 Reversion to L-form bacteria ........................................................................................... 13
2.4.5 Enzymatic activities
2.4.6 Arginine hydrolysis
2.4.7 Urea hydrolysis
2.4.8 Tetrazolium reduction
2.4.9 Tellurite reduction
2.4.10 Carbohydrate metabolism

2.5 Genetic analyses
2.5.1 DNA extraction
2.5.2 PCR amplification of phylogenetic marker genes and an intergenic spacer region
2.5.3 Cloning of 16S-23S rRNA intergenic spacer region PCR amplicons
2.5.4 Agarose gel electrophoresis of PCR products
2.5.5 Purification and sequencing of PCR amplicons
2.5.6 PCR-RFLP analysis

2.6 Protein analyses
2.6.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
2.6.2 Western Blot ("Immunoblot")

3 Results
3.1 Cell and colony morphology
3.2 Physiology and metabolism
3.3 Genetic analyses
3.4 Western blot analysis

4 Discussion

5 Summary
5.1 Abstract
5.2 Zusammenfassung .......................................................................................................................... 50

6 List of abbreviations .................................................................................................................. 51

7 Bibliography ................................................................................................................................ 54

8 List of figures and tables ............................................................................................................ 63
1 Introduction

1.1 Mycoplasmas

1.1.1 Taxonomy

Mycoplasmas are bacteria of the class *Mollicutes* (division *Tenericutes*). The word *Mollicutes* is derived from the Latin words *mollis* and *cutis*, meaning soft skin. Members of this class are small bacteria lacking a cell-wall as they do not synthesize precursors of peptidoglycan.

The class *Mollicutes* is subdivided into four orders: *Entomoplasmatales, Acheloplasmatales, Anaeroplasmatales* and *Mycoplasmatales* (Brown 2011).

The order *Mycoplasmatales* contains the family *Mycoplasmataceae*, which is divided into two genera, namely *Mycoplasma* and *Ureaplasma*.

1.1.2 Ecology and habitat

Members of the genus *Mycoplasma* (trivial name mycoplasmas) are commensals, pathogens, and opportunists in a vast range of hosts. Fish, reptiles, birds, aquatic and terrestrial mammals including humans may be colonized or infected (Razin 1992). Mycoplasmas are mainly found on mucous surfaces of the respiratory and urogenital tracts, but may also be present in/on eyes, mammary glands, joints, and serous membranes of animals and humans (Razin et al. 1998).

Mycoplasmas are considered to be highly host-specific, but some species may exhibit a wider host range than previously assumed (Pitcher and Nicholas 2005). One of these *Mycoplasma* species is *Mycoplasma (M.) arginini* which have been found in a broad range of hosts including cats, dogs, small ruminants, cattle, humans and non-human primates (Barile et al. 1968).

Mesophilic temperatures are required for their growth, a temperature range from 20 to 45 °C being tolerated by some species, but optimum growth at 37 °C is common. Most mycoplasmas are aerobic or facultative anaerobic, but some species prefer or even require anaerobic conditions, such as *M. muris* (Brown 2011).
1.1.3 Morphology

Mycoplasmas are the smallest self-replicating organisms with sizes usually varying between 300 and 800 nm. Due to the lack of a rigid cell wall mycoplasmas are pleomorphic exhibiting variability in size and shape depending on extrinsic and intrinsic factors such as the growth phase, osmotic pressure, and nutrient supply from the culture medium. The most common appearance is spherical, coccoid or flask-shaped, but cells may also form rings, disks or filaments (Bredt 1994).

Colonies are generally less than 1 mm in diameter typically exhibiting a fried-egg appearance in transmitted light (Brown 2011). The darker central zone originates from the extension of the colony into the growth medium (Quinn et al. 2011). Cauliflower-like or smooth surfaces may also be found. The margins may be smooth, irregular or scalloped differing on the species, culture medium, nutritional quality of the medium, and other extrinsic factors (Brown 2011).

Many mycoplasmas are non-motile, but some species can move on surfaces such as M. mobile, M. pneumoniae and M. gallisepticum. A prominent extension of the cytoplasm and cell membrane, a terminal organelle, is born by the organism’s cytoskeleton. This extension provides gliding motility and adherence to different surfaces due to adhesion proteins (Brown 2011).

The absence of a cell wall combined with their minute size leads to a considerable plasticity of mycoplasmas that allows filterability through 450 nm pores. Some species are even able to pass through 220 nm or 100 nm filters (Brown 2011). Due to their lack of a cell wall, mycoplasmas are resistant to lysis by lysozyme and fragile to lysis by osmotic shock or many agents provoking the lysis of bacterial protoplasts (Razin 1979, 1983).

1.1.4 Physiology and metabolism

Mycoplasmas count among the most fastidious prokaryotes regarding nutritional requirements. This stringent demand on nutrients results from their narrow capacity for intermediary metabolism, making them highly dependent upon their host. The respiratory
system is limited as all species lack a complete tricarboxylic acid cycle and do not possess quinones or cytochromes, which makes oxidative phosphorylation impossible.

To generate ATP, several non-fermentative *Mycoplasma* species hydrolyze arginine, which additionally produces ammonia, resulting in a rise of the medium’s pH. Most fermentative species gain ATP by catabolizing glucose or other carbohydrates and produce acid as a by-product, consequently lowering the pH of the medium. Species like *M. fermentans* are capable of both fermentative and non-fermentative pathways, whereas none of these two pathways exist in *M. bovis, M. agalactiae* and other species (Brown 2011). To gain energy, these species are able to oxidize pyruvate, lactate or acetate (Miles 1992, Taylor et al. 1994).

Mycoplasmas require cholesterol for growth (Razin and Tully 1970) incorporating it into the membrane’s lipids (Razin 1967). Their cell membrane is a single unit membrane consisting of amphipathic lipids and proteins (McElhaney 1992, Smith 1992, Wieslander et al. 1992). In general, cholesterol may only be found in membranes of eukaryotic cells, being the major sterol in animal cell membranes (Pollard et al. 2007), mycoplasmas therefore represent an exceptional group among prokaryotes.

A “film and spots” reaction may be found in some species, for example *M. gallinarum*, when cultivated on a medium containing heat-inactivated horse serum. After several days of incubation at 37° C, a greyish film with dark spots around the colonies may be observed (Edward 1950a). The film is composed of cholesterol and phospholipids, whereas the spots contain salts of fatty acids (Brown 2011).

Mycoplasmas exhibit an intrinsic resistance to antibiotics targeting the synthesis of cell-wall components and of folic acid since mycoplasmas lack a cell wall and do not synthesize folic acid.

1.1.5 *In vitro* cultivation

The difficulty of *in vitro* cultivation of mycoplasmas has been a major impediment to mycoplasma research and laboratory diagnosis in the past decades. Genetic explanations for these difficulties have been provided by recent mycoplasma genomic projects. Their minute genome size makes mycoplasmas totally dependent on the exogenous supply of several
metabolites such as amino acids (Himmelreich et al. 1996) and cholesterol (Razin and Tully 1970).

To overcome the restricted self-sufficiency of mycoplasmas, complex media have been developed for their cultivation. Commercially available media are usually based on beef heart infusion, peptone, yeast extract, serum and other supplements required for the successful cultivation of mycoplasmas (Razin and Hayflick 2010). Additionally, penicillin and thallous acetate are supplemented to inhibit the growth of cell-walled bacteria and fungi, respectively (Quinn et al. 2011).

Commonly used culture media for mycoplasmas are Hayflick’s medium (Hayflick 1965), Friis medium, Frey medium, and SP4 medium (Tully 1995).

### 1.1.6 Genome composition and phylogeny

Since 1995, when the first complete genome of a *Mycoplasma* species (*M. genitalium*) has been successfully sequenced (Fraser et al. 1995), the genomic database of mycoplasmas grew steadily, with currently over 80 species fully sequenced (https://www.ncbi.nlm.nih.govgenome).

Genomes of mycoplasmas are circular double-stranded and have sizes between 580 and 1350 kilobases (kb). This minute genome size, due to successive gene losses during evolution, turns mycoplasmas into organisms possessing the smallest genome size among self-replicating prokaryotes (Brown 2011).

A main characteristic of mycoplasmas is their low G+C content of 23.8-40 mol% (Brown 2011), even lying below the theoretical minimally required 26 mol% G+C in order to code for proteins with a normal amino acid composition (Razin 1985).

Many mycoplasmas contain mobile genetic elements such as integrative and conjugative elements or insertion sequence elements (Breton et al. 2012).

An exceptional characteristic of all mycoplasmas is the use of UGA to code for tryptophan, first shown in 1985 for *M. capricolum* (Yamao et al. 1985). Interestingly, this property is
shared by mitochondria (Razin 1985). Other bacteria use UGA as stop codon and UGG codes for tryptophan.

The 16S rRNA gene is an essential marker for mycoplasma phylogeny and serves together with phenotypic characteristics as a tool for taxonomy (Razin et al. 1998). The less highly conserved 16S-23S intergenic spacer region may resolve intraspecific or interspecific relationships between closely related species (Harasawa et al. 2000, Chalker and Brownlie 2004). To further increase the accuracy and reliability of mycoplasma phylogeny, the RNA polymerase β-subunit (rpoB) gene has been used as a complementary phylogenetic marker (Kim et al. 2003, Volokhov et al. 2012).

1.2 Aim of the project

Characterisation and classification of undefined Mycoplasma isolates are significant tools for epidemiologic studies in veterinary medicine. Especially in wildlife, the presence and epidemiology of mycoplasmas remain unclear as a result of different reasons such as restricted access to samples, delay in post-mortem investigation, purview ambiguity and inadequate in situ lab facilities.

The isolates characterised were collected from different hosts such as ground squirrel, cormorant, ostrich, seal, stork, and others. Characterisation and classification of the isolates were performed using genetic as well as phenotypic tests including morphologic, physiologic, serologic and metabolic analyses.

Identification and differentiation of unknown mycoplasmas are important in order to understand their impact on the health of the potential host. The aim was to analyse the isolates to accomplish an ultimate characterisation of the microorganisms allowing a valid description of the unclassified mycoplasmas in the future.
2 Material and method

2.1 Mycoplasma strains

2.1.1 *Mycoplasma* isolates

Forty-one *Mycoplasma* isolates have been investigated. The isolates were collected from various hosts and sampling sites (Tab. 1). All *Mycoplasma* isolates were stored in SP4 broth at -80 °C. The isolates were grouped according to their host species. Sixteen isolates were chosen as representatives (in bold in Tab. 1).

Tab. 1 *Mycoplasma isolates from different hosts*. Designation, host species, sampling site and year of isolation. Representatives in bold.

<table>
<thead>
<tr>
<th>Isolate designation</th>
<th>Host species</th>
<th>Isolation site</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>St57</td>
<td>White stork</td>
<td>Choana</td>
<td>2006</td>
</tr>
<tr>
<td>St93</td>
<td>White stork</td>
<td>Choana</td>
<td>2006</td>
</tr>
<tr>
<td>Sp22</td>
<td>White stork</td>
<td>Air sac</td>
<td>2005</td>
</tr>
<tr>
<td>BA019806</td>
<td>Great cormorant</td>
<td>Choana</td>
<td>2009</td>
</tr>
<tr>
<td>BA020830</td>
<td>Great cormorant</td>
<td>Choana</td>
<td>2009</td>
</tr>
<tr>
<td>BA019817</td>
<td>Great cormorant</td>
<td>Choana</td>
<td>2009</td>
</tr>
<tr>
<td><strong>BA019827</strong></td>
<td><strong>Great cormorant</strong></td>
<td><strong>Choana</strong></td>
<td><strong>2009</strong></td>
</tr>
<tr>
<td>20848/1</td>
<td>Great cormorant</td>
<td>Choana</td>
<td>2009</td>
</tr>
<tr>
<td>19836/1</td>
<td>Great cormorant</td>
<td>Choana</td>
<td>2009</td>
</tr>
<tr>
<td><strong>Moneda</strong></td>
<td><strong>South American sea lion</strong></td>
<td><strong>Lung abscess</strong></td>
<td><strong>2012</strong></td>
</tr>
<tr>
<td>Kelo</td>
<td>South American sea lion</td>
<td>Tonsil</td>
<td>2013</td>
</tr>
<tr>
<td><strong>Ralle19</strong></td>
<td><strong>Water rail</strong></td>
<td><strong>Choana</strong></td>
<td><strong>2008</strong></td>
</tr>
<tr>
<td>Ralle3</td>
<td>Water rail</td>
<td>Choana</td>
<td>2008</td>
</tr>
<tr>
<td>Ralle12</td>
<td>Water rail</td>
<td>Choana</td>
<td>2008</td>
</tr>
<tr>
<td>Sample Code</td>
<td>Species</td>
<td>Organ</td>
<td>Year</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------</td>
<td>-----------</td>
<td>-------</td>
</tr>
<tr>
<td>Ralle24</td>
<td>Water rail</td>
<td>Choana</td>
<td>2008</td>
</tr>
<tr>
<td>13/09</td>
<td>Water rail</td>
<td>Choana</td>
<td>2008</td>
</tr>
<tr>
<td>EA139239</td>
<td>Water rail</td>
<td>Choana</td>
<td>2008</td>
</tr>
<tr>
<td>EA162684</td>
<td>Water rail</td>
<td>Choana</td>
<td>2008</td>
</tr>
<tr>
<td>1052</td>
<td>Humboldt penguin</td>
<td>Lung</td>
<td>2003</td>
</tr>
<tr>
<td>1123</td>
<td>Humboldt penguin</td>
<td>Lung</td>
<td>2003</td>
</tr>
<tr>
<td>2068 K2</td>
<td>Humboldt penguin</td>
<td>Lung</td>
<td>2003</td>
</tr>
<tr>
<td>1802</td>
<td>Humboldt penguin</td>
<td>Lung</td>
<td>2003</td>
</tr>
<tr>
<td>2445/1</td>
<td>Goose</td>
<td>Phallus</td>
<td>2015</td>
</tr>
<tr>
<td>1654/K13</td>
<td>Goose</td>
<td>Phallus</td>
<td>2015</td>
</tr>
<tr>
<td>2445/5</td>
<td>Goose</td>
<td>Phallus</td>
<td>2015</td>
</tr>
<tr>
<td>1579</td>
<td>European ground squirrel</td>
<td>Lung</td>
<td>2015</td>
</tr>
<tr>
<td>3686</td>
<td>European ground squirrel</td>
<td>Lung</td>
<td>2015</td>
</tr>
<tr>
<td>Zaradi2</td>
<td>Cattle</td>
<td>Semen</td>
<td>2002</td>
</tr>
<tr>
<td>Haberl</td>
<td>Cattle</td>
<td>Semen</td>
<td>2008</td>
</tr>
<tr>
<td>2282</td>
<td>Cattle</td>
<td>Semen</td>
<td>2014</td>
</tr>
<tr>
<td>310</td>
<td>Cattle</td>
<td>Semen</td>
<td>2011</td>
</tr>
<tr>
<td>1331</td>
<td>Cattle</td>
<td>Semen</td>
<td>2010</td>
</tr>
<tr>
<td>291</td>
<td>Cattle</td>
<td>Semen</td>
<td>2007</td>
</tr>
<tr>
<td>Gleisdorf25</td>
<td>Cattle</td>
<td>Semen</td>
<td>2006</td>
</tr>
<tr>
<td>AA1</td>
<td>Spanish imperial eagle</td>
<td>Trachea</td>
<td>2006</td>
</tr>
<tr>
<td>M13mK1</td>
<td>Spanish imperial eagle</td>
<td>Choana</td>
<td>2006</td>
</tr>
<tr>
<td>HF6</td>
<td>Bonelli’s eagle</td>
<td>Choana</td>
<td>2006</td>
</tr>
<tr>
<td>HF8/C</td>
<td>Bonelli’s eagle</td>
<td>Choana</td>
<td>2006</td>
</tr>
</tbody>
</table>
2.1.2 Control strains

Fifteen *Mycoplasma* species have been used as control strains for various tests (Tab. 2).

<table>
<thead>
<tr>
<th>Species</th>
<th>Type strain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma agalactiae</em></td>
<td>PG2&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma alkalescens</em></td>
<td>PG51&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma arthritidis</em></td>
<td>PG6&lt;sup&gt;I&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma bovirhinis</em></td>
<td>PG43&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma bovis</em></td>
<td>PG45&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma buteonis</em></td>
<td>Bb/T2g&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma canis</em></td>
<td>PG14&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma caviae</em></td>
<td>G122&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma cynos</em></td>
<td>H831&lt;sup&gt;I&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma edwardii</em></td>
<td>PG24&lt;sup&gt;I&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma fermentans</em></td>
<td>PG18&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma gallinarum</em></td>
<td>PG16&lt;sup&gt;I&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma galopavonis</em></td>
<td>WR1&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma mycoides ssp. mycoides</em></td>
<td>PG1&lt;sup&gt;I&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycoplasma primatum</em></td>
<td>HRC292&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
2.2 Cultivation of mycoplasmas

A small portion (approximately 100 µl) of each frozen isolate (-80 °C) was transferred to 4 ml SP4 broth and incubated at 37 °C for 24 to 48 hours. Cultures exhibiting a colour change from orange to yellow or red and/or increased turbidity of the medium were diluted in SP4 broth (1:1000) and 100 µl of dilution were plated on SP4 agar. Agar plates were incubated at 37 °C in 7 % CO₂ atmosphere until isolated colonies were observed. One colony was picked, transferred into 4 ml SP4 broth and further cultivated at 37 °C. Aliquots (500 µl) of cultures were stored at -80 °C until further usage.

2.2.1 SP4 medium

The SP4 medium was prepared as follows.

- **SP4 broth**

  PPLO<sup>a</sup> Broth (BD Difco™, Austria) | 2.5 g  
  Tryptone (BD Difco™, Austria) | 7 g  
  Peptone G (BD Difco™, Austria) | 3.5 g  
  DNA (Sigma-Aldrich, Austria) | 0.14 g  
  dH₂O | 700 ml  

<sup>a</sup>pleuropneumonia-like organisms

Adjust the pH to 7.4.

Autoclave at 121 °C for 15 min.

Cool medium to 50 °C and add:

Heat-inactivated horse serum (Biowest, France) | 50 ml  
Heat-inactivated swine serum (Biowest, France) | 50 ml  
Heat-inactivated fetal bovine serum (Biowest, France) | 20 ml  
Glucose, 50 % solution (Roth, Germany) | 7 ml
L-arginine, 50 % solution (Sigma-Aldrich, Austria) 3.5 ml
CMRL\textsuperscript{a} 1066, 10 x concentrated (Thermo Scientific Gibco\textsuperscript{TM}, Austria) 35 ml
Yeast Extract, 4 % solution (Oxoid, Austria) 35 ml
NAD\textsuperscript{b}, 1 % solution (Roth, Germany) 11 ml
L-cysteine, 1 % solution (Sigma-Aldrich, Austria) 17 ml
Penicillin (Sandoz, Austria) 1,000,000 I.U.
Thallium acetate, 10 % solution (Sigma-Aldrich, Austria) 1.4 ml
Phenol red, 0.6 % solution (Sigma-Aldrich, Austria) 4 ml
\textsuperscript{a}Connaught Medical Research Laboratories \textsuperscript{b}β-Nicotinamide adenine dinucleotide
Sterile tubes were filled with 4 ml of the medium and stored at 7 °C.

- **SP4 agar:**

SP4 agar medium was prepared analogue to the SP4 broth with an addition of 8.5 g purified agar (Oxoid, Austria) after adjustment of the pH.

### 2.2.2 PPLO base medium

PPLO base medium was used for the following tests: casein hydrolysis, fermentation of pyruvate, reduction of tetrazolium and tellurite and metabolism of carbohydrates. The medium was prepared as follows.

- **PPLO broth base:**

PPLO Broth (BD Difco\textsuperscript{TM}, Austria) 21 g
dH\textsubscript{2}O 700 ml

Autoclave at 121 °C for 15 min.

Cool medium to 50-60 °C and add:
Yeast Extract, 25 % solution (Merck, Germany)  85 ml

Heat-inactivated horse serum (Biowest, France)  170 ml

Penicillin (Sandoz, Austria)  1,000,000 I.U.

If needed, phenol red was added to the medium:

Phenol red, 1 % solution (Sigma-Aldrich, Austria)  40 ml

- **PPLO agar base:**

  PPLO Broth (BD Difco™, Austria)  21 g
  
dH2O  700 ml
  
Agar Noble (BD Difco™, Austria)  9.8 g

Autoclave at 121 °C for 15 min.

Cool medium to 50-60 °C and add:

Yeast Extract, 25 % solution (Merck, Germany)  85 ml

Heat-inactivated horse serum (Biowest, France)  170 ml

Penicillin (Sandoz, Austria)  1,000,000 I.U.

### 2.3 Cell and colony morphology

#### 2.3.1 Colony morphology

The colony morphology of 16 *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) was examined at x 40 magnification using a stereo microscope (Olympus, Germany). SP4
agar plates were inoculated with 100 \( \mu \)l of broth cultures and incubated at 37 °C in 7 % CO\(_2\) atmosphere until typical mycoplasma colonies were visible.

### 2.3.2 Filtration

Filterability was tested on 16 *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) using membrane filters with pore sizes of 450 nm and 220 nm (Roth, Germany).

### 2.4 Physiology and metabolism

#### 2.4.1 Optimum growth temperature and oxygen requirement

Sixteen *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) were investigated on SP4 agar.

The optimum growth temperature was tested under aerobic conditions at 28, 37 and 45 °C. Additionally, cultivation was performed under aerobic and anaerobic conditions to test whether the isolates are strictly aerobic or facultative anaerobic. Plates were therefore incubated in jars with gas packs providing anaerobic (Anaerocult A, Merck, Germany) or microaerobic conditions (CO\(_2\) generator, BD BBL™, Austria).

#### 2.4.2 Film and spots

The production of film and spots, first described in 1950 by Edward, was studied after an extended incubation (approximately seven days) of isolates St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802 and HF8/C at 37 °C on SP4 agar. The following strains were used as controls (Aluotto et al. 1970):

Positive: *M. gallinarum* PG16\(^T\)

Negative: *M. arthritidis* PG6\(^T\)
2.4.3 Digitonin sensitivity

Sixteen *Mycoplasma* isolates were tested for sensitivity to digitonin (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) (Whitford et al. 1994). Filter disks with digitonin were prepared as follows:

- **Digitonin (Sigma-Aldrich, Austria)** 15 mg
- **Ethanol 95 % (Sigma-Aldrich, Austria)** 1 ml

Dispense on filter disks, 25 µl each.

Dry overnight at 37°C.

SP4 agar plates were inoculated with 100 µl SP4 broth cultures and a filter disk containing digitonin were placed in the center of the inoculated plate. Plates were cultivated at 37 °C for 24-48 hours. A positive reaction was indicated by a clear zone of growth inhibition around the disc.

2.4.4 Reversion to L-form bacteria

L-form bacteria also lack a rigid cell wall and may, therefore, simulate mycoplasmas. To differentiate mycoplasmas from L-form bacteria Columbia blood agar plates (BD, Austria) were inoculated with 100 µl SP4 broth cultures of 16 *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) and colony morphology was evaluated after 24-48 hours of incubation at 37 °C. The presence of a typical colony appearance (< 1mm) identified the organisms as a species of the class *Mollicutes*.

2.4.5 Enzymatic activities

Semi-quantification of enzymatic activities was performed using the API ZYM test kit (Biomérieux, France). The following 19 enzymes were tested: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, mannosidase, fucosidase.
Sixteen *Mycoplasma* isolates were investigated (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C).

Isolates were cultivated in SP4 broth at 37 °C for 24-48 hours. One ml of broth culture was then centrifuged at 20,000 x g for 10 min. The supernatant was removed, and the pellet was washed twice with 500 µl 0.9 % saline. After centrifugation at 20,000 x g the pellet was mixed with 1.5 ml saline and transferred to the wells of the API ZYM test kit which was incubated in a moist chamber at 37 °C for approximately four hours. To develop the chromogenic substrates two reagents, A and B, were used. Reagent A contained (per 100 ml) 25 g Tris, 11 ml HCl (37 %), and 10 g sodium dodecyl sulfate. Reagent B consisted of 0.35 % Fast Blue BB salt in 2-methoxyethanol. Colour reactions were induced by adding one drop of each reagent to each well. After 5 to 15 min, color intensities were evaluated and classified using the following scale: “-” for no activity, “(-)” for low activity, “(+)” for moderate activity, and “+” for high activity of the enzyme. A color chart provided by the manufacturer was used as a reference.

### 2.4.6 Arginine hydrolysis

SP4 broth and agar used for the cultivation of all *Mycoplasma* isolates (Tab.1) contained arginine. After incubation at 37 °C for 24-48 hours, a colour shift from orange to red or magenta was observed if the isolate was capable to hydrolyse arginine.

### 2.4.7 Urea hydrolysis

U4 agar plates (Institute of Microbiology, University of Veterinary Medicine, Austria) were used to test for the capability of isolates to hydrolyze urea. The plates were incubated for 24-48 hours at 37 °C and 7 % CO₂ atmosphere. Sixteen *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) were tested. Urea hydrolysis was indicated if a colour change of the agar medium from yellow to pink or magenta was observed.

### 2.4.8 Tetrazolium reduction

To test for the capability of isolates to reduce tetrazolium, the following agar plates were prepared:
Plates were inoculated by inverted agar blocks (SP4 agar) with colonies as described by Aluotto et al. (1970). Each of the 16 *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802, HF8/C) was incubated at 37 °C anaerobically using a jar with gas packs (Anaerocult A, Merck, Germany). Plates were incubated up to two weeks and controlled regularly.

Development of a pink or red colour where colonies have been transferred constituted a positive reaction. This phenomenon is based on the reduction of tetrazolium to insoluble formazan (Aluotto et al. 1970).

The following strains served as controls:

- Positive: *M. bovirhinis* PG43<sup>T</sup>
- Negative: *M. arthritidis* PG6<sup>T</sup>

### 2.4.9 Tellurite reduction

To test for the capability of isolates St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 13/09, 20 (C3), 1802 and HF8/C to reduce tellurite, the following agar plates were prepared:

- PPLO Agar Base (Chapter 2.2.2) 100 ml
- Potassium tellurite, 1 % solution (Sigma-Aldrich, Austria) 0,5 ml

Identical procedures of inoculation and incubation as described above for tetrazolium reduction were performed.

Black coloured colonies indicated a positive reaction (Aluotto et al. 1970).
The following strains were included as controls:

Positive: *M. bovirhinis* PG43<sup>T</sup>
Negative: *M. arthritidis* PG6<sup>T</sup>

### 2.4.10 Carbohydrate metabolism

Fermentation of the following 49 carbohydrates was tested using the API 50 CH test kit (Biomérieux, France): glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xyopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidone, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate.

*Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 2371A, BA019827, 13/09, 20 (C3), 1802, HF8/C) were cultivated in PPLO Broth Base containing phenol red at 37 °C for 24-48 hours.

For the inoculation of the testing strips, a 10 ml ampule of API 50 CHB/E medium was inoculated with the cultivated PPLO Broth Base. The API 50 CHB/E medium is composed of 2 g ammonium sulfate, 0.5 g yeast extract, 1 g tryptone, 3.22 g disodium phosphate, 0.12 g monopotassium phosphate, 10 ml trace elements, 0.17 g phenol red, and 1000 ml demineralized water. A suspension with a turbidity equivalent to 2 McFarland was prepared using a densitometer. The tubes of the testing strips were then inoculated with the suspension and were incubated in a moist chamber at 37 °C for up to 48 hours.

The strips were checked for a positive reaction after 24 and 48 hours of incubation. A positive reaction was indicated by a colour change from red to yellow except for esculin where a black colourization revealed a positive reaction.
2.5 Genetic analyses

2.5.1 DNA extraction

DNA of 18 *Mycoplasma* isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 171, 13/09, 20 (C3), 1802, HF8/C, EA139239) was extracted using UltraClean® Tissue & Cells DNA Isolation Kit (MO BIO Laboratories, USA). The kit contained tubes with dry beads, and solutions TD1, TD2 and TD3, proteinase K solution, spin filters and 2 ml collection tubes.

Cell pellets harvested by centrifugation of 1 ml broth cultures at 14,000 x g for 10 min, were resuspended in 600 µl of solution TD1 and mixed thoroughly. The solution was transferred to tubes containing dry beads and mixed with a shaker for 10 min. Tubes were then placed in a thermomixer at 60 °C overnight.

Tubes were then centrifuged at 10,000 x g for 10 min and the entire supernatant was transferred to a spin filter which was centrifuged at 10,000 x g for 30 seconds. The flow through was discarded, 400 µl of solution TD2 were added and spin filters were centrifuged at 10,000 x g for 30 seconds. The flow through was again discarded and another centrifugation at 10,000 x g for 1 min was conducted to remove residual solution TD2. Spin filters were placed in a new 2 ml collection tube and 50 µl of solution TD3 was added to the centre of the filter membrane. After centrifugation at 10,000 x g for 30 seconds, spin filters were discarded and DNA in the collection tube was stored at -20 °C until further use.

2.5.2 PCR amplification of phylogenetic marker genes and an intergenic spacer region

2.5.2.1 Material for PCR

OneTaq® Quick-Load® DNA Polymerase (OneTaq) (New England BioLabs®, USA) was used to perform PCR amplification. All primers were purchased from Invitrogen (Thermo Scientific, Austria) and are listed in Tab. 3. Primers were used in a 10 pm/µl concentration. A Mastercycler® nexus (Eppendorf, Germany) was used for PCR amplification.
**Tab. 3 Primers used for PCR amplification.** Target gene, primer names, primer sequences and references.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer sequences</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>27f</td>
<td>5’-AGA GTT TGA TCM TGG CTC AG-3’</td>
<td>(Lane 1991)</td>
</tr>
<tr>
<td></td>
<td>1492r</td>
<td>5’-TAC GGY TAC CTT GTT ACG ACT T-3’</td>
<td>(Lane 1991)</td>
</tr>
<tr>
<td>16S-23S intergenic spacer region</td>
<td>F2A</td>
<td>5’-GTG GGG ATG GAT CAC CTC CT-3’</td>
<td>(Tang et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>5’-GCA TCC ACC AAA AAC TCT T-3’</td>
<td>(Tang et al. 2000)</td>
</tr>
<tr>
<td></td>
<td>ITSavian-F</td>
<td>5’-CGT TCT CGG GTC TTG TAC AC-3’</td>
<td>(Ramírez et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>ITSavian-R</td>
<td>5’-CGC AGG TTT GCA CGT CCT TCA TCG-3’</td>
<td>(Ramírez et al. 2008)</td>
</tr>
<tr>
<td>Cloned amplicon</td>
<td>Cloning Analysis F Primer (Clon-F)</td>
<td>5’-ACC TGC CAA CCA AAG CGA GAA C-3’</td>
<td>NEB® PCR Cloning KitΔ</td>
</tr>
<tr>
<td></td>
<td>Cloning Analysis R Primer (Clon-R)</td>
<td>5’-TCA GGG TTA TTG TCT CAT GAG CG-3’</td>
<td>NEB® PCR Cloning KitΔ</td>
</tr>
<tr>
<td>rpoB</td>
<td>rpoB-F-MYC</td>
<td>5’-AGT TAT CAC AAT TTA TGG ATC AAA-3’</td>
<td>(Volokhov et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>rpoB-R-MYC</td>
<td>5’-GCT CAH ACT TCC ATT TCH CCA AA-3’</td>
<td>(Volokhov et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>MPF1</td>
<td>5’-GAY ATT GAT CAC TTA GGT AA-3’</td>
<td>(Kim et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>MPF2</td>
<td>5’-AGA TGA TGA YCC NGA TTC A-3’</td>
<td>(Kim et al. 2003)</td>
</tr>
</tbody>
</table>

Δ: New England BioLabs®, USA.
2.5.2.2 Amplification of the 16S rRNA gene

The partial 16S rRNA gene of 17 Mycoplasma isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1,13/09, HF8(TypC), 2FIA, 237IA, BA019827, 20(C3), 1802, EA139239) were amplified using primers 27f and 1492r (Lane 1991). The reaction mixture for the amplification using PCR is described in Tab. 4.

Tab. 4 Reaction mixture for 16S rRNA gene amplification. Component and volume.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>OneTaq</td>
<td>12,5 µl</td>
</tr>
<tr>
<td>Primer 27f</td>
<td>0,75 µl</td>
</tr>
<tr>
<td>Primer 1492r</td>
<td>0,75 µl</td>
</tr>
<tr>
<td>Template</td>
<td>2,5 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>ad 25 µl</td>
</tr>
</tbody>
</table>

PCR amplification was performed at 95 °C for 5 min, followed by 30 cycles at 94 °C for 2 min, 50 °C for 1.5 min, and 72 °C for 5 min. Amplification was finished by a final extension at 72 °C for 1 min.

2.5.2.3 Amplification of the 16S-23S intergenic spacer region (ISR)

Amplification of the 16S-23S rRNA intergenic spacer region (ISR) was performed for 16 Mycoplasma isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 13/09, HF8(TypC), 2FIA, 237IA, BA019827, 20(C3), 1802 and EA139239) using two different pairs of primers (Tab. 3). F2A and R2 were used for all isolates, ITSavian-F and ITSavian-R were used for nine isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, AA1, BA019827). Reaction mixtures are shown below (Tab. 5, Tab. 6).
Tab. 5 Reaction mixture using primers F2A and R2. Component and volume.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>OneTaq</td>
<td>25 µl</td>
</tr>
<tr>
<td>Primer F2A</td>
<td>5 µl</td>
</tr>
<tr>
<td>Primer R2</td>
<td>5 µl</td>
</tr>
<tr>
<td>Template</td>
<td>2,5 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>ad 50 µl</td>
</tr>
</tbody>
</table>

Amplification was accomplished at 94 °C for 30 sec, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min.

Tab. 6 Reaction mixture using primers ITSavian-F and ITSavian-R. Component and volume.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>OneTaq</td>
<td>12,5 µl</td>
</tr>
<tr>
<td>Primer ITSavianF</td>
<td>0,5 µl</td>
</tr>
<tr>
<td>Primer ITSavianR</td>
<td>0,5 µl</td>
</tr>
<tr>
<td>Template</td>
<td>2,5 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>ad 25 µl</td>
</tr>
</tbody>
</table>

Amplification was performed at 94 °C for 15 sec, followed by 35 cycles at 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 2 min, and a final elongation at 72 °C for 5 min.

2.5.2.4 Amplification of the rpoB gene

The rpoB gene, encoding the β-subunit of RNA polymerase, was partially amplified in 17 Mycoplasma isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 2FIA, 237IA, BA019827, 171, 13/09, 20 (C3), 1802, HF8/C). Primers used were three
different forward primers (rpoB-F-MYC, MPF1 and MPF2) and one reverse primer (rpoB-R-MYC) (Tab. 3). For all 17 isolates, the primer pair rpoB-F-MYC and rpoB-R-MYC were used. Only the \( \textit{rpoB} \) gene of isolate St57 was additionally amplified with MPF1 or MPF2 as forward primers and rpoB-R-MYC as reverse primer as unsatisfying results were obtained employing primer pair rpoB-F-MYC and rpoB-R-MYC. The reaction mixture for PCR was identical for all primer pairs (Tab. 7).

**Tab. 7 Reaction mixture for partial \( \textit{rpoB} \) gene amplification.** Forward primers used: rpoB-F-MYC, MPF1 and MPF2. Reverse primer used: rpoB-R-MYC.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>OneTaq</td>
<td>30 µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>2,5 µl</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>2,5 µl</td>
</tr>
<tr>
<td>Template</td>
<td>2,5 µl</td>
</tr>
<tr>
<td>( \text{ddH}_2 \text{O} )</td>
<td>ad 60 µl</td>
</tr>
</tbody>
</table>

Amplification was performed at 95 °C for 6 min followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 68 °C for 2 min, and a final extension at 68 °C for 5 min.

2.5.3 Cloning of 16S-23S rRNA intergenic spacer region PCR amplicons

16S-23S rRNA ISR amplicons of isolates 1052, 1579 and HF8/C were cloned since sequencing revealed heterogeneous sequences in these isolates. For cloning the NEB® PCR Cloning Kit (New England BioLabs®, USA) was used. The Cloning Kit contained the following components: Cloning mix 1 and 2, linearized pMiniTTM vector, amplicon cloning control (1 kb), cloning analysis forward (Clon-F) and reverse primer (Clon-R), NEB 10-beta competent \( \textit{Escherichia coli} \) (\( \textit{E. coli} \)) (Cloning Efficiency), pUC19 control DNA and SOC outgrowth medium.

Cloning was performed according to the manufacturer’s instruction as following (Tab. 8).
• **Ligation**

**Tab. 8 Formula for the ligation mixture.** Ligation reaction for isolate HF8/C was prepared with 2 µl, for the other two isolates 1 µl insert was added.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearized pMiniT Vector (25 ng/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Insert</td>
<td>1 µl or 2 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>ad 5 µl</td>
</tr>
<tr>
<td>Cloning mix 1</td>
<td>4 µl</td>
</tr>
<tr>
<td>Cloning mix 2</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

The first four components were mixed together before cloning mix 1 and 2 were added. The mixture was incubated at room temperature for 15 min, followed by incubation on ice for 2 min.

• **Transformation**

50 µl of competent *E. coli* cells were thawed on ice for 10 min and 2 µl of ligation reaction were added and mixed gently by flicking the tube 4-5 times. The mixture was incubated on ice for 20 min, heat shocked at 42 °C for 30 sec and subsequently cooled on ice for 5 min. 950 µl of SOC medium were added and the solution was placed at 37 °C for 60 min on an orbital shaker at 250 rpm (outgrowth step)

• **Plating**

The following agar plates containing ampicillin were prepared:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone (Roth, Germany)</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast Extract, 25% solution (Merck, Germany)</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl (Roth, Germany)</td>
<td>10 g</td>
</tr>
</tbody>
</table>
Agar Noble (Oxoid, Austria) 15 g

\[ \text{dH}_2\text{O ad 1000 ml} \]

The solution was autoclaved and cooled down to approximately 50 °C, before ampicillin was added to a final concentration of 100 µg/ml.

Cells gained from the successful outgrowth step were mixed thoroughly and 50 µl of the outgrowth, as well as 50 µl of a 1:10 dilution prepared with SOC, were spread on agar plates containing ampicillin. Plates were incubated overnight at 37 °C.

- **Screening**

Screening for inserts was performed by colony PCR (Tab. 9).

**Tab. 9 Reaction mixture using primers Clon-F and Clon-R.** Primers were part of the NEB® PCR Cloning Kit (New England BioLabs®, USA).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>OneTaq</td>
<td>25 µl</td>
<td></td>
</tr>
<tr>
<td>Primer Clon-F</td>
<td>0.15 µl</td>
<td></td>
</tr>
<tr>
<td>Primer Clon-R</td>
<td>0.15 µl</td>
<td></td>
</tr>
<tr>
<td>ddH\textsubscript{2}O</td>
<td>ad 50 µl</td>
<td></td>
</tr>
</tbody>
</table>

The template was added by picking an individual colony from the inoculated agar plates.

PCR was performed under the following conditions: Initial denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 15 sec, 53 °C for 15 sec and 68 °C for 60 sec, and a final extension at 68 °C for 5 min.
2.5.4 Agarose gel electrophoresis of PCR products

Separation of the PCR amplicons was performed in a 1.5 % (w/v) agarose gel. The agarose gel was prepared by dissolving agarose (Sigma-Aldrich, Austria) in heated 1 x TAE buffer. The 1 x TAE buffer was obtained by dilution of a 50 x TAE buffer stock.

- 50 x TAE buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS&lt;sup&gt;a&lt;/sup&gt; (Roth, Germany)</td>
<td>242 g</td>
</tr>
<tr>
<td>Glacial acetic acid (Roth, Germany)</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>0.5 M EDTA&lt;sup&gt;b&lt;/sup&gt;, pH 8</td>
<td>100 ml</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>ad 1000 ml</td>
</tr>
</tbody>
</table>

<sup>a</sup>tris(hydroxymethyl)aminomethane, <sup>b</sup>ethylenediaminetetraacetic acid

Gel electrophoresis was performed in an electrophoresis chamber. 7 µl of the PCR amplification products and 4 µl of a molecular weight marker (NEB<sup>®</sup> 2-Log DNA Ladder, New England BioLabs<sup>®</sup>, USA) were loaded onto the agarose gel. Gels were stained in a 2.5 mg/l ethidium bromide solution for approximately 20 min. Amplicons were visualized using a ChemiDoc<sup>TM</sup> MP Imaging System (Bio-Rad Laboratories, Germany).

2.5.5 Purification and sequencing of PCR amplicons

PCR amplicons were purified using GeneJET PCR Purification Kit (Thermo Scientific, Austria). The purification kit contained a binding buffer, washing buffer, elution buffer (10 mM TRIS-HCl, pH 8.5), and purification columns. Purification was performed as described by the manufacturer.

Binding buffer was mixed 1:1 with the PCR product. In case of the amplicon being ≤ 500 bp, a 1:2 volume of 100% isopropanol was added. Up to 800 µl of the mixture were then transferred into a purification column and centrifuged at 6,700 x g for 60 sec. The flow-through was discarded and 700 µl of washing buffer were added to the column. After
centrifugation at 6,700 x g for 60 sec, the flow-through was again discarded and the purification column was placed back into the collection tube. To assure the complete removal of residual washing buffer, the purification column was additionally centrifuged at 12,000 x g for 1 min. The purification column was finally transferred to a clean 1.5 ml tube and 50 µl of the elution buffer were added to the centre of the purification column membrane and centrifuged at 6,700 x g for 1 min. Purified amplicons were stored at -20 °C until further usage.

Purified PCR products were sent to LGC Genomics, Berlin, Germany, for sequencing amplicons in both directions.

### 2.5.6 PCR-RFLP analysis

For PCR-restriction fragment length polymorphism (RFLP) analysis PCR products of the 16S-23S intergenic spacer region of all 16 Mycoplasma isolates (St57, BA019806, Moneda, Ralle19, 1052, 2445/1, 1579, Zaradi2, AA1, 13/09, HF8(TypC), 2FIA, 237IA, BA019827, 20(C3), 1802 and EA139239) were digested as described previously (Spergser and Rosengarten 2007) using the restriction endonucleases TaqI, VspI, ApoI (Fermentas, Germany) and DdeI (Segenetic, Germany).

Digestion of each amplicon was performed using both combinations. 4 µl of the PCR product were added to a mixture of 36 µl 10x buffer (18 µl of each buffer if two were combined) and 4 µl of the endonucleases (2 µl of each enzyme, 10,000 units/ml). The digestion mixtures were incubated overnight at 37 °C and electrophoresed in a 1.5% (w/v) agarose gel. The agarose gel was stained in a 2.5 mg/L ethidium bromide solution for 20 min prior to visualization under UV light. Restriction fragment sizes were determined by comparison to NEB® 2-Log DNA Ladder (New England BioLabs®, USA).
2.6 Protein analyses

The immunogenic protein profiles of the following seven isolates representing a new *Mycoplasma* species of cattle were studied: Zaradi2, Haberl, 2282, 310, 1331, 291, Gleisdorf25. In addition, profiles of type strains of the three closely related *Mycoplasma* species, *M. bovis*, *M. agalactiae*, and *M. primatum*, were generated and compared to those of the seven isolates.

2.6.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Mycoplasma cells were harvested from 3 ml of log-phase broth cultures (SP4) by centrifugation at 12,000 x g for 5 min. Pellets were resuspended in 260 µl or 360 µl phosphate-buffered saline (PBS) depending on the size of the pellet. 52-72 µl of SDS loading sample buffer were added to the solution. Proteins were denaturised by heating at 95 °C for 5 min.

- **2x SDS loading sample buffer, stored at -20 °C**

  0,5 M TRIS\(^a\)-HCl, pH 7,0 (Sigma-Aldrich, Austria) 2.5 ml
  Glycerol, 87 % (v/v) (Roth, Germany) 2 ml
  SDS\(^b\), 10 % (w/v) (Roth, Germany) 4 ml
  2-mercaptoethanol (Sigma-Aldrich, Austria) 1 ml
  bromophenol blue (Sigma-Aldrich, Austria) 15 mg
  dH\(_2\)O 0.5 ml

\(^a\)tris(hydroxymethyl)aminomethane, \(^b\)sodium dodecyl sulfate

- **Separating gel 10 % (w/v)**

  Acrylamide/Bis 29:1, 40 % (w/v) (Bio Rad Laboratories, Germany) 10 ml
  1,5 M TRIS\(^a\)-HCl, pH 8,8 (Sigma-Aldrich, Austria) 10 ml
  SDS\(^b\), 10 % (w/v) (Roth, Germany) 0.4 ml
  Ammonium persulfate, 10 % (w/v) (Sigma-Aldrich, Austria) 0.4 ml
TEMED\textsuperscript{c} (Sigma-Aldrich, Austria) 16 µl
dH\textsubscript{2}O ad 40 ml

\textsuperscript{a}tris(hydroxymethyl)aminomethane, \textsuperscript{b}sodium dodecyl sulfate, \textsuperscript{c}tetramethylethylenediamine

- **Stacking gel**

Acrlyamide/Bis 29:1, 40 % (w/v) (Bio Rad Laboratories, Germany) 1.25 ml

1,0 M TRIS\textsuperscript{a}-HCl, pH 6,8 (Sigma-Aldrich, Austria) 1.25 ml

SDS\textsuperscript{b}, 10 % (w/v) (Roth, Germany) 0.1 ml

Ammonium persulfate, 10 % (w/v) (Sigma-Aldrich, Austria) 0.1 ml

TEMED\textsuperscript{c} (Sigma-Aldrich, Austria) 10 µl
dH\textsubscript{2}O ad 10 ml

\textsuperscript{a}tris(hydroxymethyl)aminomethane, \textsuperscript{b}sodium dodecyl sulfate, \textsuperscript{c}tetramethylethylenediamine

Electrophoresis was performed using an electrophoresis chamber. The buffer used for the electrophoresis was prepared as described by Laemmli (1970). The gel was loaded with 30 µl of each protein extracts and 7 µl of molecular weight marker (Page Ruler\textsuperscript{TM} Unstained Protein Ladder, Thermo Scientific, Austria). Proteins were separated at 130 V and electrophoresis was stopped before the dye reached the end of the gel.

2.6.2 **Western Blot (“Immunoblot”)**

Western blotting is an immunologic method for the detection of immunoreactive proteins separated by SDS-PAGE. Proteins first need to be transferred from the polyacrylamide gel onto a nitrocellulose membrane, before antibodies can bind to the proteins.
2.6.2.1 Protein transfer onto a nitrocellulose membrane

Transfer of proteins from the polyacrylamide gel onto a nitrocellulose membrane was completed using a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio Rad Laboratories, Germany). Proteins were transferred in Towbin buffer at 14 V and 180 mA for approximately one hour.

- Towbin buffer

  TRIS* base (Sigma-Aldrich, Austria) 3.03 g
  Glycine (Roth, Germany) 14.4 g
  dH₂O 500 ml
  Methanol (Sigma-Aldrich, Austria) 300 ml
  dH₂O ad 1000 ml

  *tris(hydroxymethyl)aminomethane

3.7.2.2 Detection of proteins using hyperimmune sera

After the transfer of the proteins, blots were incubated at room temperature for one hour with a solution of bovine serum albumin to block unspecific binding sites. Thereafter, blots were washed three times with PBS-Tween®-20 for 15 min. Blots were incubated with rabbit hyperimmune serum raised against isolates investigated (1:1000 diluted in PBS) overnight at 4 °C. The rabbit hyperimmune serum was assembled in 2008 at the former Institute for Bacteriology, Mycology and Hygiene at the University of Veterinary Medicine in Vienna, following standard protocols (Brown et al. 2006). Afterwards, blots were again washed with PBS- Tween®-20 three times for 15 min. The membrane was then incubated for two hours with a secondary antibody (anti-rabbit IgG-HRP, Sigma-Aldrich, Austria) at room temperature. Then, blots were washed three times for five minutes using PBS. Finally, a substrate solution containing chloronaphthol was added for staining purposes. Staining was stopped by adding tap water immediately after colouring was seen.
- **BSA\(^a\) solution**

  BSA\(^a\) (Sigma-Aldrich, Austria) 3 g
  PBS\(^b\) (Roth, Germany) 100 ml

  \(^a\)bovine serum albumin, \(^b\)phosphate-buffered saline

- **Chloronaphthol solution**

  HRP\(^a\) Colour Development Reagent 12 mg containing chloronaphthol (Bio Rad Laboratories, Gemany)
  Methanol (Sigma-Aldrich, Austria) 4 ml
  PBS\(^b\) (Roth, Germany) 20 ml
  H\(_2\)O\(_2\), 30 % (v/v) (Sigma-Aldrich, 20 µl Austria)

  \(^a\)horseradish peroxidase, \(^b\)phosphate-buffered saline

  The HRP Colour Development Reagent was first diluted in methanol and then mixed with PBS. H\(_2\)O\(_2\) was added to the solution directly before use.

- **PBS-Tween**

  Tween\(^®\)-20 (Merck, Germany) 1 ml
  PBS\(^a\) (Roth, Germany) ad 1000 ml

  \(^a\)phosphate-buffered saline
3 Results

3.1 Cell and colony morphology

For all 16 isolates, direct light microscopy of the untreated SP4 agar plates revealed a typical “fried-egg” appearance, describing a dense central button (DCB) within a colony (Fig. 1). In AA1 a disappearance of the DCB was observed after four days of cultivation and the colonies gained a diffuse brown colouration (Fig. 1 b). Aside from strain Moneda, colonies of all isolates exhibited smooth and entire margins (Fig. 2 a). Strain Moneda (Fig. 2 b) displayed a peripheral lacy pattern with discontinued margins and colonies of strain 1052 (Fig. 2 c) had a granular appearance. A vast divergence in colony sizes was observed and varied from 25 µm to 500 µm in diameter, with strain St57 exhibiting the largest colonies (Tab. 10). Most of the isolates (11 out of 16) showed non-uniform colony sizes, with a difference up to 225 µm in diameter. Additionally, nearly half of the isolates (7 out of 16) showed a more or less pronounced confluent growth.

![Fig. 1 Colonies of AA1 on SP4 agar medium at x 40 magnification after 24 hours (a) and 96 hours (b) of cultivation. In (a) the “fried egg” appearance is existent whereas in (b) the central dense button has vanished and a diffuse brown colouration of the colony. Scale: 100 µm.](image)

Cells of AA1 and 13/09 were the only cells not able to pass a membrane filter with a pore diameter of 220 nm. They were therefore tested for their filterability through a 450 nm
membrane filter, showing cells of 13/09 were neither filterable through this pore size, whereas cells of AA1 were able to pass it.

Fig. 2 Colonies of BA019806 (a), Moneda (b) and 1052 (c) on SP4 agar medium at x 40 magnification. Scale: 100µm.
**Tab. 10 Size and uniformity of colonies.** Isolate, uniformity of the colony size and colony size in µm. The values indicated represent the average diameter of the colonies.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Uniformity</th>
<th>Colony size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>St57</td>
<td>uniform</td>
<td>500</td>
</tr>
<tr>
<td>BA019806</td>
<td>uniform</td>
<td>200</td>
</tr>
<tr>
<td>Moneda</td>
<td>non-uniform</td>
<td>100-300</td>
</tr>
<tr>
<td>Ralle19</td>
<td>uniform</td>
<td>100</td>
</tr>
<tr>
<td>1052</td>
<td>non-uniform</td>
<td>50-250</td>
</tr>
<tr>
<td>2445/1</td>
<td>non-uniform</td>
<td>25-200</td>
</tr>
<tr>
<td>1579</td>
<td>non-uniform</td>
<td>50-250</td>
</tr>
<tr>
<td>Zaradi2</td>
<td>uniform</td>
<td>250</td>
</tr>
<tr>
<td>AA1</td>
<td>non-uniform</td>
<td>150-250</td>
</tr>
<tr>
<td>13/09</td>
<td>uniform</td>
<td>350</td>
</tr>
<tr>
<td>HF8/C</td>
<td>non-uniform</td>
<td>100-250</td>
</tr>
<tr>
<td>2FIA</td>
<td>non-uniform</td>
<td>150-250</td>
</tr>
<tr>
<td>237IA</td>
<td>non-uniform</td>
<td>50-200</td>
</tr>
<tr>
<td>BA019827</td>
<td>non-uniform</td>
<td>50-200</td>
</tr>
<tr>
<td>20 (C3)</td>
<td>non-uniform</td>
<td>25-300</td>
</tr>
<tr>
<td>1802</td>
<td>non-uniform</td>
<td>50-200</td>
</tr>
</tbody>
</table>
### 3.2 Physiology and metabolism

For most of the tested isolates, the mean duration of growth at 37°C under aerobic conditions was 24 hours (h). The only exemptions were strain 1579 and Zaradi2 with a growth duration of 48h and 72h, respectively.

Test for oxygen requirement revealed only strain Moneda as an obligate aerobe as no growth was seen under anaerobic conditions. In some cases, growth under anaerobic conditions was slower than under aerobic conditions, suggesting that these organisms’ ability to produce ATP in absence of oxygen is less efficient compared to their ATP yield in an aerobic environment. No isolate was found to be an obligate anaerobe.

The production of film and spots was present in 4 isolates: St57, 1052, 2445/1 and BA019827. At 37°C and 42°C with aerobic conditions St57 and BA019827 exhibited formation of film and spots after an incubation time of 5 days and 3 days, respectively. 1052 and 2445/1 only showed a film and spot reaction at a cultivation temperature of 42°C, with an incubation time of 6 days and 3 days, respectively.

For all the isolates, testing for the capability of urea hydrolysis and reversion to L-form bacteria was negative (strain 2445/1 showed no growth on Columbia blood agar plates), and sensitivity to digitonin was present in all isolates. Unsurprisingly, the premise that all the isolates belong to the genus *Mycoplasma* was therefore confirmed.

The cultivation on Columbia blood agar plates (BD, Austria) revealed the ability of α- and β-hemolysis (Tab. 11) distributed as follows.

**Tab. 11 Type of hemolysis.** Isolate, type of hemolysis. “α” α-hemolysis; “β” β-hemolysis.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Type of hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>St57</td>
<td>α</td>
</tr>
<tr>
<td>BA019806</td>
<td>α</td>
</tr>
<tr>
<td>Moneda</td>
<td>α</td>
</tr>
<tr>
<td>Ralle19</td>
<td>β</td>
</tr>
<tr>
<td>1052</td>
<td>β</td>
</tr>
</tbody>
</table>
Ten isolates were capable to hydrolyze arginine: St57, St93, 1052, 2068 K2, 1123237IA, 19836/1, 20848/1, Sp22, 1802.

The test for tellurite reduction was positive for all isolates except for HF8/C. Positive isolates showed a black colourization of colonies (Fig. 3 a). Eleven out of sixteen Mycoplasma isolates were capable to reduce tetrazolium to formazan (Fig. 3 b): St57, BA019806, Moneda, Ralle19, 1052, 1579, Zaradi2, 2FIA, 237IA, BA019827 and 13/09; resulting in a red colourization of colonies. In neither of the two reduction tests growth of 2445/1 has been observed.

![Mycoplasma colonies on SP4 agar medium at x 40 magnification. Black colourisation of colonies after reduction of tellurite (a) and red coloured colonies after reduction of tetrazolium to formazan (b). Scale: 500 µm.](image)

**Fig. 3** Mycoplasma colonies on SP4 agar medium at x 40 magnification. Black colourisation of colonies after reduction of tellurite (a) and red coloured colonies after reduction of tetrazolium to formazan (b). Scale: 500 µm.
Evaluation of the API ZYM test kit (Biomérieux, France) revealed the activity of twelve enzymes (Tab. 12). A positive reaction for sixteen substrates could be demonstrated using the API 50 CH test kit (Biomérieux, France) (Tab. 13).
### Tab. 12 Results of the API ZYM test kit

Isolate, enzyme tested, result. Meaning of the scale used: “-“, no activity; “(-)”, low activity; “(+)”, moderate activity; and “+”, high activity.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>ALP</th>
<th>EST</th>
<th>ESL</th>
<th>LAA</th>
<th>VAA</th>
<th>APH</th>
<th>NPH</th>
<th>AGL</th>
<th>BGL</th>
<th>ABG</th>
<th>MAN</th>
<th>FUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>St57</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BA019806</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Moneda</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ralle19</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(-)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1052</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2445/1</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1579</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zaradi2</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(-)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13/09</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HF8(TypC)</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>(-)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2FIA</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(-)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2371A</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BA019827</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20(C3)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1802</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Tests with negative results for all isolates have been excluded. ALP, alkaline phosphatase; EST, esterase; ESL, esterase lipase; LAA, leucine arylamidase; VAA, valine arylamidase; APH, acid phosphatase; NPH, naphthol-AS-B1-phosphohydrolase; AGL, α-glucosidase; BGL, β-glucosidase; ABG, N-acetyl-β-glucosaminidase; MAN, mannosidase; FUC, fucosidase.*
Tab. 13 Results of the API 50 CH test kit\textsuperscript{a}. Isolate, substrate tested, result. Meaning of the scale used: “-“, no reaction; “(-)”, low reaction; “(+)”, moderate reaction; and “+”, strong reaction.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>GLY</th>
<th>DAR</th>
<th>LAR</th>
<th>RIB</th>
<th>DXY</th>
<th>LXY</th>
<th>GLU</th>
<th>MNE</th>
<th>NAG</th>
<th>MAL</th>
<th>LAC</th>
<th>AMD</th>
<th>GLYG</th>
<th>TUR</th>
<th>LYX</th>
<th>2KG</th>
<th>5KG</th>
</tr>
</thead>
<tbody>
<tr>
<td>St57</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>BA019806</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Moneda</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ralle19</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1052</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>2445/1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>1579</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Zaradi2</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>13/09</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>HF8(TypC)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>2FIA</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(-)</td>
<td>(-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>237IA</td>
<td></td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>BA019827</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>20(C3)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Test with negative results for all isolates have been excluded. GLY; glycerol; DAR, D-arabinose; LAR, L-arabinose; RIB, D-ribose; DXY, D-xylose; LXY, L-xylose; GLU, D-glucose; MNE, D-mannose; NAG, N-acetyl-glucosamine; MAL, D-maltose; LAC, lactose; AMD, amidone; GLYG, glycogen; TUR, D-turanose; LYX, D-lyxose; 2KG, 2-ketogluconate; 5KG, 5-ketogluconate.
3.3 Genetic analyses

After PCR-RFLP analysis of the 16S-23S rRNA intergenic spacer region (Fig. 4), eleven groups of identical profiles have been identified. Interestingly, identical isolates shared the same host species (except for group 9 with isolates originating from different animal species of the same genus), suggesting a strong host specificity of these *Mycoplasma* species (Tab. 14).

![Fig. 4](image-url) Restriction enzyme digestion of the 16S-23S rRNA intergenic spacer region. Group A BA020830; group B BA019817; group C BA019806. Lane 1 16S-23S intergenic spacer region; lane 2 *TaqI* and *VspI* digest; lane 3 *ApoI* and *DdeI* digest. M molecular weight marker (NEB® 2-Log DNA Ladder (New England BioLabs®, USA)).
**Tab. 14 Groups of identical PCR-RFLP profiles.** Group, isolates, animal species or genus of origin.

<table>
<thead>
<tr>
<th>Group</th>
<th>Isolates</th>
<th>Species/genus of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>St57; St93</td>
<td>White stork</td>
</tr>
<tr>
<td>2</td>
<td>BA019806; BA019817; BA020830</td>
<td>Great cormorant</td>
</tr>
<tr>
<td>3</td>
<td>Moneda; Kelo; 3968/11</td>
<td>South American sea lion</td>
</tr>
<tr>
<td>4</td>
<td>Ralle19; Ralle3; Ralle12; Ralle24</td>
<td>Water rail</td>
</tr>
<tr>
<td>5</td>
<td>1052; 2068 K2; 1123</td>
<td>Humboldt penguin</td>
</tr>
<tr>
<td>6</td>
<td>2445/1; 1654/K13; 2445/5</td>
<td>Goose</td>
</tr>
<tr>
<td>7</td>
<td>1579; 3686</td>
<td>European ground squirrel</td>
</tr>
<tr>
<td>8</td>
<td>Zaradi2; 2282; Gleisdorf25; Haberl; 291; 310;1331</td>
<td>Cattle</td>
</tr>
<tr>
<td>9</td>
<td>AA1; HF6; M13mK1</td>
<td>True eagles&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>13/09; EA139239; EA162684</td>
<td>Water rail</td>
</tr>
<tr>
<td>11</td>
<td>BA019827; 19836/1; 20848/1</td>
<td>Great cormorant</td>
</tr>
</tbody>
</table>

<sup>a</sup>Genus, Latin: *Aquila*.

Amplicons of two genes and one intergenic spacer region (16S rRNA gene, 16S-23S intergenic spacer region, and *rpoB*) were sequenced and subjected to similarity search against the GenBank nucleotide database using BLAST (https://blast.ncbi.nlm.nih.gov/) and highest similarity values were determined (Tab. 15).
Tab. 15 Highest similarity values of the 16S rRNA gene, the 16S-23S intergenic spacer region, and of partial *rpoB* gene. Isolate, closest relatives (indicated by similarity values in %).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>16S rRNA gene</th>
<th>16S-23S ISR</th>
<th>partial <em>rpoB</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>St57</td>
<td><em>M. gypis</em> (97)</td>
<td><em>M. gypis</em> (86)</td>
<td>no amplicon obtained</td>
</tr>
<tr>
<td>BA019806</td>
<td><em>M. buteonis</em> (97)</td>
<td><em>M. buteonis</em> (90)</td>
<td><em>M. glycophilum</em> (87)</td>
</tr>
<tr>
<td>Moneda</td>
<td><em>M. edwardii</em> (97)</td>
<td><em>M. felis</em> (89)</td>
<td><em>M. edwardii</em> (82)</td>
</tr>
<tr>
<td>Ralle19</td>
<td><em>M. columborale</em> (97)</td>
<td><em>M. columborale</em> (89)</td>
<td><em>M. columborale</em> (87)</td>
</tr>
<tr>
<td>1052</td>
<td><em>M. felifaecium</em> (97)</td>
<td><em>M. felifaecium</em> (95)</td>
<td><em>M. felifaecium</em> (89)</td>
</tr>
<tr>
<td>2445/1</td>
<td><em>M. fermentans</em> (94)</td>
<td><em>M. fermentans</em> (79)</td>
<td><em>M. fermentans</em> (84)</td>
</tr>
<tr>
<td>1579</td>
<td><em>M. citelli</em> (98)</td>
<td><em>M. columborale</em> (88)</td>
<td><em>M. citelli</em> (88)</td>
</tr>
<tr>
<td>Zaradi2</td>
<td><em>M. primatum</em> (97)</td>
<td><em>M. primatum</em> (88)</td>
<td><em>M. primatum</em> (84)</td>
</tr>
<tr>
<td>AA1</td>
<td><em>M. anatis</em> (96)</td>
<td><em>M. sturni</em> (84)</td>
<td><em>M. gallinaceum</em> (87)</td>
</tr>
<tr>
<td>13/09</td>
<td><em>M. pullorum</em> (96)</td>
<td><em>M. pullorum</em> (83)</td>
<td><em>M. pullorum</em> (83)</td>
</tr>
<tr>
<td>HF8(TypC)</td>
<td><em>M. anatis</em> (95)</td>
<td><em>M. felis</em> (83)</td>
<td><em>M. gallinaceum</em> (87)</td>
</tr>
<tr>
<td>2FIA</td>
<td><em>M. verecundum</em> (95)</td>
<td><em>M. verecundum</em> (78)</td>
<td><em>M. verecundum</em> (84)</td>
</tr>
<tr>
<td>2371A</td>
<td><em>M. spumans</em> (98)</td>
<td><em>M. falconis</em> (91)</td>
<td><em>M. neophronis</em> (84)</td>
</tr>
<tr>
<td>BA019827</td>
<td><em>M. maculosum</em> (98)</td>
<td><em>M. maculosum</em> (92)</td>
<td><em>M. maculosum</em> (87)</td>
</tr>
<tr>
<td>20(C3)</td>
<td><em>M. canis</em> (96)</td>
<td><em>M. felis</em> (89)</td>
<td><em>M. edwardii</em> (84)</td>
</tr>
<tr>
<td>1802</td>
<td><em>M. spumans</em> (96)</td>
<td><em>M. phocidae</em> (91)</td>
<td><em>M. neophronis</em> (84)</td>
</tr>
<tr>
<td>EA139239</td>
<td><em>M. pullorum</em> (96)</td>
<td><em>M. pullorum</em> (82)</td>
<td><em>M. pullorum</em> (82)</td>
</tr>
</tbody>
</table>
3.4 Western blot analysis

Western blot analysis of whole-cell lysates of Zaradi2, Haberl, 2282, 310, 1331, 291, Gleisdorf25, *M. primatum* HRC292\(^T\), *M. agalactiae* PG2\(^T\) and *M. bovis* PG45\(^T\) were performed using anti-*Mycoplasma* sp. Zaradi2 antiserum. Blots revealed identical immunogenic banding patterns within the seven isolates which clearly differed from those profiles observed for *M. primatum* HRC292\(^T\), *M. agalactiae* PG2\(^T\) and *M. bovis* PG45\(^T\) (Fig. 5).

![Blot of whole-cell lysates of M. primatum HRC292T (1), M. agalactiae PG2T (2), M. bovis PG45T (3), Gleisdorf25 (4), 291 (5), 1331 (6), 310 (7), 2282 (8), Haberl (9), and Zaradi2 (10) after incubation with anti-Mycoplasma sp. Zaradi2 antiserum. Immunogenic banding patterns of isolates 4 to 10 are identical and a clear discrimination to the profiles of Mycoplasma species 1 to 3 is apparent.](image)

**Fig. 5** Blot of whole-cell lysates of *M. primatum* HRC292T (1), *M. agalactiae* PG2T (2), *M. bovis* PG45T (3), Gleisdorf25 (4), 291 (5), 1331 (6), 310 (7), 2282 (8), Haberl (9), and Zaradi2 (10) after incubation with anti-Mycoplasma sp. Zaradi2 antiserum. Immunogenic banding patterns of isolates 4 to 10 are identical and a clear discrimination to the profiles of *Mycoplasma* species 1 to 3 is apparent.
4 Discussion

In this study, a polyphasic characterisation including phenotypic criteria such as cultural, morphological, biochemical and serological characteristics, as well as molecular genetics of unknown *Mycoplasma* isolates was performed with the purpose to obtain a fundamental characterisation enabling a valid description of the unclassified organisms in the future.

Based on phenotypic criteria including a typical umbonate ("fried egg"-type) appearance of the colonies, digitonin sensitivity, the absence of reversion to walled cells, and lack of capability to hydrolyze urea all 16 unique isolates have been identified as members of the genus *Mycoplasma*.

A high number of identical isolates originating from the same host species have been revealed through molecular genetic tests. This prominent association between identical isolates and host suggests strong host specificity among these *Mycoplasma* species.

Currently, the classification of *Mollicutes* species is based on few phenotypic features but the required tests are time-consuming and labor-intensive and results may be hampered by phenotypic variations among the isolates (Volokhov et al. 2012) Additionally, only few biochemical reactions are applicable for phenotypic characterisation of members of the class *Mollicutes*, therefore phenotypic identification strongly relies on serological characteristics (Pollack et al. 1996, Pollack et al. 1997, Knight Jr. 2004, Volokhov et al. 2007). Nevertheless, data gained from phenotypic analyses may be utilized to test the assumptions on the phylogenetic placement after serological analyses and 16S rRNA gene sequencing (Brown et al. 2007).

Available tests for metabolic and enzymatic features only provide limited discriminatory power for species identification of members of the family *Mycoplasmataceae*. Assignment of isolates to either the genus level or fermentation group (i.e. glucose-, urea-, or arginine-utilizers) may be the only attainable interpretation of biochemical analyses (Volokhov et al.
2012). Thus, the use of biochemical tests may solely aim the characterisation of an isolate but does not offer a valuable basis for species identification and differentiation.

Westernblot analysis was performed on one group of identical isolates (i.e. Zaradi2, 2282, Gleisdorf 25, Haberl, 291, 310, 1331) and three *Mycoplasma* species with closely related to this group expressing 16S rRNA gene similarity values $>97\%$ (i.e. *M. primatum*, *M. bovis*, and *M. agalactiae*). Results showed a clear distinction between the banding pattern of the isolates and the known *Mycoplasma* species and an identical protein pattern among the isolates.

From the earliest days of mycoplasmology, serological characteristics represented the major framework for species identification and description of novel species. However, due to the presence of potential interspecies cross-reactivity of antibodies in hyperimmune sera made to the type strain of each species and considerable serologic heterogeneity of some species, serological misidentification and ambiguous discrimination of isolates cannot be completely eliminated (Brown et al. 2007, Volokhov et al. 2012). Moreover, there are no commercially available antisera to *Mollicutes* and correct performance and accurate interpretation of these tests require considerable experience and skill (Volokhov et al. 2007, 2012). Despite the difficulties that may be encountered, serological tests still constitute a crucial tool in defining species of the class *Mollicutes* according to the revised minimal standards proposed by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of Mollicutes (Brown et al. 2007).

Additionally, uncultivated and fastidious species of *Mycoplasmataceae* hamper, or even make impossible, the use of conventional bacteriological tests. These circumstances made molecular genetic analyses an essential additional tool for primary identification and phylogenetic classification of both cultivable and uncultivable or fastidious *Mycoplasmataceae* (Volokhov et al. 2012). Analysis of genetic markers may be performed in standard microbiological laboratories and do not require specific skill in mycoplasmology. The information collected from molecular genetic tests may be employed for the choice of
appropriate metabolic and serological assays for the final classification of isolates (Volokhov et al. 2012).

The 16S rRNA gene is the most commonly used molecular marker in bacterial taxonomy because of its exceptionally slow rate of evolution (Volokhov et al. 2007). Over the past 20 years, 16S rRNA gene sequencing provided a revolution in bacterial taxonomy and created uniformity among the traditional identification schema for the domain *Bacteria* (Clarridge 2004, Schloss and Handelsman 2004, Janda and Abbott 2007, Yarza et al. 2008, Volokhov et al. 2012). By using different molecular biological techniques such as sequencing (Gray et al. 2005, Nicolas et al. 2005), denaturing gradient gel electrophoresis (McAuliffe et al. 2005) and restriction digestion (Stakenborg et al. 2005) the 16S rRNA gene represents a valuable complementary marker for species identification (Volokhov et al. 2006). It has been recommended for phylogenetically based assignment of new species of the class *Mollicutes* in 2007 and evolved to one of the mandatory requirements for the description of new *Mollicutes* species (Brown et al. 2007, Volokhov et al. 2012).

The minimum level of the interspecies sequence similarity value for 16S rRNA, indicating a genetically distant species, was proposed to be ≤ 97 %, based on gene data gained through phylogenetic studies of closely related species (Pettersson et al. 2000). Hence, in the case of two strains from the same cluster or group having 16S rRNA sequence similarity greater than 97 %, it is likely that they belong to the same species. Nevertheless, sequence similarity values above 97 % may also be found among closely related well-known *Mycoplasma* species which demonstrate differences in serological, further genetic and ecological features that allow defining them as individual species (Volokhov et al. 2012).

However, not all difficulties of the genetic identification of a species can be solved by using the 16S rRNA gene as a solitary universal genetic marker, especially in cases of close relatedness between organisms of the same genus (Blackwood et al. 2004). Furthermore an intragenomic heterogeneity of 16S rRNA genes has been demonstrated in several studies (Nübel et al. 1996, Wang et al. 1997, Yap et al. 1999) causing an overestimation of microbial diversity (Crosby and Criddle 2003, Case et al. 2007, Sun et al. 2013). For these ambiguous
situations where an accurate identification of the species cannot be provided, a polyphasic taxonomical approach, which considers all suitable phenotypic and genetic tests, might yield a definitive species identification of isolates (Vandamme et al. 1996, Stackebrandt et al. 2002).

In 2012, Volokhov et al. proposed the partial nucleotide sequences of the RNA polymerase β-subunit gene (\textit{rpoB}) and the 16S-23S rRNA intergenic spacer region (ISR) as complementary phylogenetic markers to derive phylogenetic relationships among the \textit{Mycoplasmataceae} species. Acceptance criteria for universal genetic markers used for the genetic identification of a large number of bacterial species include high enough levels of inter-species diversity and low intra-species polymorphism, further an efficient PCR amplification of these genes and identification of the organisms of interest to the species level. Both the 16S-23S rRNA ISR gene and the partial \textit{rpoB} gene have been validated to meet these criteria (Ludwig and Schleifer 1994, Gürtler und Stanisch 1996, Volokhov et al. 2007, 2012, Adékambi et al. 2009).

In the study of Volokhov et al. (2012), comparison of the 16S-23S rRNA ISR and \textit{rpoB} phylogenetic trees with the referential phylogenetic tree of the 16S rRNA gene for the \textit{Mycoplasmataceae} species showed similar clustering patterns. Thus Volokhov et al. (2012) reasoned a three-target sequence analysis including the 16S-23S rRNA ISR, \textit{rpoB} and 16S rRNA genes to be a reliable and effective taxonomic tool for the species differentiation within the \textit{Mycoplasmataceae} family based on phylogenetic relationship and pairwise sequence similarities. An arbitrary value to define a new species of 95-98 $\%$ similarity for the 16S-23S rRNA ISR sequence and 90-91 $\%$ similarity for the \textit{rpoB} gene were proposed by Volokhov et al. (2012). The similarity value suggested for the \textit{rpoB} gene does exclude members of the mycoides cluster, for which a similarity value of 97-98 $\%$ similarity was found to be more accurate due to a high level of interspecies similarities (Volokhov et al. 2012).

Thus, a similarity value of 95 $\%$ similarity of sequences of the 16S-23S rRNA ISRs of isolate 1052 and \textit{M. felisfaucium} (Tab. 15) either suggests a close relatedness of these species or affiliation of the isolate to the \textit{M. felisfaucium} species. The result of the 16S rRNA gene
similarity search for isolate 1052, showing a sequence similarity of 97 % with *M. felisfauclium*, supports this theory. Similarity values of the 16S-23S rRNA ISRs of the other isolates were below the threshold proposed by Volokhov et al. (2012).

Similarity values of the 16S rRNA gene of nine isolates (St57, BA019806, Moneda, Ralle19, 1052, 1579, Zaradi2, 237IA, BA019827) were 97 % or higher, but none of the other two marker genes (16S-23S rRNA ISR and partial *rpoB* gene) analysed showed sequence similarities above the threshold, except for 1052 as discussed before. Partial *rpoB* gene sequences of all isolates exhibited similarity values below 90-91 % similarity.

Results of the similarity search against the GenBank nucleotide database using BLAST (https://blast.ncbi.nlm.nih.gov/) (Tab. 15) point to the assumption that all isolates investigated represent new species, some with high level similarities of the 16S rRNA gene sequence to known *Mycoplasma* species. Only isolate 1052 might belong to the *M. felisfauclium* species according to the similarity values obtained for the 16S rRNA gene and 16S-23S rRNA ISR.

An arbitrary value to define a new species of 95-9% similarity for the 16S-23S rRNA ISR sequence and 90-9% similarity for the *rpoB* gene were proposed by Volokhov et al. (2012). The similarity value suggested for the *rpoB* gene does exclude members of the mycoides cluster, for which a similarity value of 97-9% similarity was found to be more accurate due to a high level of interspecies similarities (Volokhov et al. 2012).

A new path in diagnostic microbiology and taxonomic investigation of micro-organisms is the usage of Matrix-Assisted Laser Desorption Ionisation Flight-of-Time (MALDI-ToF) Mass Spectrometry. (MS) introduced in the 1980s to 1990s (Fox 2006). This so-called soft ionization MS technology is increasingly applied to identify routine isolates from hospital patients (Clark et al. 2013) and identification of bacterial species from animal origin has though been successfully performed in the past ten years (Randall et al. 2015). Studies showed that MALDI-ToF is a rapid, reliable, simple and inexpensive method to identify micro-organisms to the genera or even species level based on whole cell analysis (Biswaas and Rolain 2013, Randall et al. 2015, Ge et al. 2017).

Spergser et al. (2019) performed a study investigating the usability of MALDI-ToF for the identification and differentiation of almost all animal related *Mycoplasma* species isolated so
The study included 114 known and 23 undescribed taxa of mycoplasmas, representing three genera and 13 phylogenetic groups within the class *Mollicutes*. A total of 530 MSPs were collected in a large in-house library which was shown to be a highly robust database yielding reproducible and reliable results by analyzing 335 independent clinical isolates, with log scores ≥1.800 to be reliable for species identification (Spergser et al. 2019). Both Pereyre et al. (2013) and Spergser et al. (2019) advocate reduction of the species-level identification threshold for mycoplasma identification of ≥1.700 and ≥2.000 recommended by BrukerDaltonics.

Studies demonstrated that MALDI-ToF MS is an excellent method for species identification and differentiation in mycoplasmology (Pereyre et al. 2013, Spergser et al. 2019), circumventing remaining problems with the identification of certain *Mycoplasma* species due to close intraspecies relatedness and low expression of biochemical traits useful for diagnostics (Le Grand et al. 2004, Tardy et al. 2009). MALDI-ToF represents a powerful of animal mycoplasmas and may be used as a screening method for unknown mycoplasma species isolated from animals (Spergser et al. 2019).

Fast and reliable identification of clinical isolates is necessary for an accurate and rapid treatment of patients. In the last decades, time between receiving a bacterial sample and identification of the organism decreased significantly for most of the species investigated due to new technologies that work faster and with a higher repetitious accuracy. In the 1990’s taxonomical description of micro-organisms was revolutionized by molecular genetics, first and foremost by using the 16S rRNA gene as genetic marker (Woese 1987, Giovannoni et al. 1990), which became the “gold standard” in both bacterial phylogeny and microbial ecology studies. Later, alternative genetic markers, as the 16S-23S rRNA ISR gene and the partial *rpoB* gene, were recognized as useful complements to the 16S rRNA gene in order to classify bacterial organisms to the species level (Case et al. 2007, Volokhov et al. 2012). Today, a new revolution is finding its way into diagnostic laboratories, the MALDI-ToF MS. Its advantages regarding costs, labor-time and simplicity make this new technology a coequal or even superior method for bacterial identification compared to traditional tests (Biswas and Rolain 2013, Randall et al. 2015, Ge et al. 2017). Ongoing supplementation of the database for
MALDI-ToF should further improve its utility (Randall et al. 2015) and might establish it as the new “gold standard” for microbial identification.

The following study, with a widely ranged phylogenetic and phenotypic characterization of the unknown *Mycoplasma* isolates, provides a solid basis for further analyses of the veterinary related significance and the epidemiology of these presumably new *Mycoplasma* species. Further studies of these species should include genomic analyses to yield a complete and significant characterization.
5 Summary

5.1 Abstract

Forty-one unclassified Mycoplasma isolates from 14 different hosts were investigated in order to clarify their taxonomic position. Isolates were taken from various isolation sites of mainly wildlife hosts e.g. European ground squirrel, White stork, Water rail, Humboldt penguin and others.

Affiliation to genus Mycoplasma was confirmed for all isolates investigated based on specific phenotypic criteria (umbonate appearance of colonies, digitonin sensitivity, absence of reversion to walled cells, lack of capability to hydrolyse urea). Standard tests for the characterization of further phenotypic features such as colony morphology, growth temperature, filterability, carbohydrate metabolism, oxygen requirement and others were performed. In addition, amplicons of the 16S rRNA gene, the 16S-23S intergenic spacer region (ISR), and the rpoB gene were sequenced and subjected to similarity search against sequences in GenBank database using BLAST, and similarity values were determined. Results suggest that all isolates investigated represent new Mycoplasma species, excluding isolate 1052 which demonstrated borderline similarity values for both, the 16S rRNA gene and the ISR to Mycoplasma felisfacium (97 % and 95 %, respectively). ISR-PCR-RFLP analysis revealed eleven groups of identical profiles. Isolates within a profile type shared the same host species or genus, indicating high host specificity among these isolates. Furthermore, western blot analysis of whole-cell lysates of ISR-PCR-RFLP profile type 8 isolates (Zaradi2, Haberl, 2282, 310, 1331, 291, Gleisdorf25) revealed identical immunogenic banding patterns clearly differing from those observed for the closely related Mycoplasma species, M. primatum HRC292T, M. agalactiae PG2T and M. bovis PG45T.

In summary, the study results provide a solid basis for the valid description of new Mycoplasma species that have been isolated from various hosts.
5.2 Zusammenfassung


Zusammenfassend liefern die Ergebnisse der vorliegenden Studie eine solide Basis für die valide Beschreibung neuer Mykoplasmenarten, die von verschiedenen Tierarten isoliert werden konnten.
6 List of abbreviations

*M. = Mycoplasma*

Kb = kilobase

*rpoB = RNA polymerase β sub-unit*

PPLO = pleuropneumonia-like organisms

CMRL = Connaught Medical Research Laboratories

NAD = β-nicotinamide adenine dinucleotide

OneTaq = OneTaq® Quick-Load® DNA Polymerase

Clon-F = Cloning Analysis Forward Primer

Clon-R = Cloning Analysis Reverse Primer

ISR = intergenic spacer region

*E. coli = Escherichia coli*

TRIS = tris(hydroxymethyl)aminomethane

EDTA = ethylenediaminetetraacetic acid

SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

PBS = phosphate-buffered saline

SDS = sodium dodecyl sulfate

TEMED = tetramethylethylenediamine

BSA = bovine serum albumin

HRP = horseradish peroxidase

DCB = dense central button
ALP = alkaline phosphatase

EST = esterase

ESL = esterase lipase

LAA = leucine arylamidase

VAA = valine arylamidase

APH = acid phosphatase

NPH = naphthol-AS-B1-phosphohydrolase

AGL = α-glucosidase

BGL = β-glucosidase

ABG = N-acetyl-β-glucosaminidase

MAN = mannosidase

FUC = fucosidase

GLY = glycerol

DAR = D-arabinose

LAR = L-arabinose

RIB = D-ribose

DXY = D-xylose

LXY = L-xylose

GLU = D-glucose

MNE = D-mannose

NAG = N-acetyl-glucosamine
MAL = D-maltose
LAC = lactose
AMD = amidone
GLYG = glycogen
TUR = D-turanose
LYX = D-lyxose
2KG = 2-ketogluconate
5KG = 5-ketogluconate
MALDI-ToF = matrix-assisted laser desorption ionisation time of flight
MS = mass spectrometry
MSP = main spectrum profile
7 Bibliography


Razin S, Tully JG. 1970. Cholesterol requirement of mycoplasmas. Journal of Bacteriology,


8 List of figures and tables

Tab. 1 Mycoplasma isolates from different hosts. Designation, host species, sampling site and year of isolation. .............................................................................................................................................6

Tab. 2 Mycoplasma control strains used for different tests. .....................................................................................8

Tab. 3 Primers used for PCR amplification. Target genes, primer names, primer sequences and references. ...........................................................................................................................................18

Tab. 4 Reaction mixture for 16S rRNA gene amplification. Component and volume. ..............................19

Tab. 5 Reaction mixture using primers F2A and R2. Component and volume. ...........................................20

Tab. 6 Reaction mixture using primers ITSavian-F and ITSavian-R. Component and volume. ..................................................................................................................................................20

Tab. 7 Reaction mixture for partial rpoB gene amplification. Forward primers used: rpoB-F-MYC, MPF1 and MPF2. Reverse primer used: rpoB-R-MYC. ........................................................................21

Tab. 8 Formula for the ligation mixture. Ligation reaction for isolate HF8/C was prepared with 2 µl, for the other two isolates 1 µl insert was added. ..................................................................................22

Tab. 9 Reaction mixture using primers Clon-F and Clon-R. Primers were part of the NEB® PCR Cloning Kit (New England BioLabs®, USA). ........................................................................................................23

Tab. 10 Size and uniformity of colonies. Isolate, uniformity of the colony size and colony size in µm. The values indicated represent the average diameter of the colonies. ........................................32

Tab. 11 Type of hemolysis. Isolate, type of hemolysis. “α” α-hemolysis; “β” β-hemolysis. ..................33

Tab. 12 Results of the API ZYM test kit®. Isolate, enzyme tested, result. Meaning of the scale used: “-”, no activity; “(-)”, low activity; “(+)”, moderate activity; and “+”, high activity. ....36
Tab. 13 Results of the API 50 CH test kit. Isolate, substrate tested, result. Meaning of the scale used: “-”, no reaction; “(-)”, low reaction; “(+)”, moderate reaction; and “+”, strong reaction.

Tab. 14 Groups of identical PCR-RFLP profiles. Group, isolates, animal species or genus of origin.

Tab. 15 Highest similarity values of the 16S rRNA gene, the 16S-23S intergenic spacer region, and of partial rpoB gene. Isolate, closest relatives (indicated by similarity values in %).

Fig. 1 Colonies of AA1 on SP4 agar medium at x 40 magnification after 24 hours (a) and 96 hours (b) of cultivation. In (a) the “fried egg” appearance is existent whereas in (b) the central dense button has vanished and a diffuse brown colouration of the colony. Scale: 100 µm.

Fig. 2 Colonies of BA019806 (a), Moneda (b) and 1052 (c) on SP4 agar medium at x 40 magnification. Scale: 100 µm.

Fig. 3 Mycoplasma colonies on SP4 agar medium at x 40 magnification. Black colourisation of colonies after reduction of tellurite (a) and red coloured colonies after reduction of tetrazolium to formazan (b). Scale: 500 µm.

Fig. 4 Restriction enzyme digestion of the 16S-23S rRNA intergenic spacer region. Group A BA020830; group B BA019817; group C BA019806. Lane 1 16S-23S intergenic spacer region; lane 2 TaqI and VspI digest; lane 3 ApoI and DdeI digest. M molecular weight marker (NEB® 2-Log DNA Ladder (New England BioLabs®, USA)).

Fig. 5 Blot of whole-cell lysates of M. primatum HRC292T (1), M. agalactiae PG2T (2), M. bovis PG45T (3), Gleisdorf25 (4), 291 (5), 1331 (6), 310 (7), 2282 (8), Haberl (9), and Zaradi2 (10) after incubation with anti-Mycoplasma sp. Zaradi2 antiserum. Immunogenic banding patterns of isolates 4 to 10 are identical and a clear discrimination to the profiles of Mycoplasma species 1 to 3 is apparent.