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Assessment of the antibiotic sensitivity and genomic studies on waterfowl *Mycoplasma* species

Ph.D. thesis

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Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	base pair
CCU	colour changing unit
CDS	coding sequence
cgMLST	core genome multilocus sequence typing
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded deoxyribonucleic acid
G+C	guanine-cytosine
GE	genomic equivalents
ITS	intergenic transcribed spacer region
kbp	kilobase pair
M.	*Mycoplasma*
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration that inhibits 50% of the isolates
MIC_{90}	minimum inhibitory concentration that inhibits 90% of the isolates
MLST	multilocus sequence typing
MP	mate-pair
NEAC	neuraminidase enzymatic activity
No.	number
PCR	polymerase chain reaction
PE	paired-end
PGAP	Prokaryotic Genome Annotation Pipeline
RAST	Rapid Annotations using Subsystems Technology
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SNP	single nucleotide polymorphism
SRA	Sequence Read Archive
ST	sequence type
tmRNA	transfer-messenger ribonucleic acid
tRNA	transfer ribonucleic acid

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1. Summary

Mycoplasma anserisalpingitidis, *M. anatis*, *M. anseris* and *M. cloacale* colonise geese and ducks. The main symptoms in the affected flocks are inflammation of the cloaca and genital tracts, decreased egg production and increased embryo lethality. The infection can also be associated with infections of the respiratory and nervous systems. Infection of geese with *M. anserisalpingitidis* causes significant production and economic losses in the goose farming business in Hungary.

Since there is no commercially available vaccine against these species, adequate housing and appropriate antibiotic treatment are promoted to control of the disease. In this study, *in vitro* susceptibility of 38 *M. anserisalpingitidis* field isolates to thirteen different antibiotics and an antibiotic combination were determined. Most of the samples were isolated from geese from several parts of Hungary, between 2011 and 2015. Most isolates showed high minimum inhibitory concentration (MIC) values against tilmicosin, oxytetracycline, norfloxacin and difloxacin. Valnemulin, tiamulin and tylvalosin were found to be the most effective antibiotics; however, isolates with high MIC values were detected for all tested antibiotics. These results highlight the importance of testing *Mycoplasma* isolates for antibiotic susceptibility before starting the treatment.

To develop new molecular genetic methods, the knowledge of the genetic background of the studied organism is required. However, the complete genomes were not available for any of the examined waterfowl *Mycoplasma* species. The type strain of *M. anserisalpingitidis* (ATCC BAA-2147) and two clinical isolates, along with the type strains of *M. anatis*, *M. anseris* and *M. cloacale* (NCTC 10156, ATCC 49234, and NCTC 10199, respectively) were *de novo* sequenced. The complete genomes were deposited in the GenBank, providing a basis for further scientific projects.

Co-occurrence of the waterfowl *Mycoplasma* species is frequently detected and identification of these mycoplasmas at the species level in a regular microbiology laboratory is difficult due to their similar morphological, cultural and biochemical properties. Previously, species differentiation was possible by the sequence analysis of the product of a genus-specific polymerase chain reaction (PCR) assay. Therefore, PCR assays using species-specific primers were designed, which target housekeeping genes to identify the concerned species. The developed PCR assays can precisely identify *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* at the species level, directly from DNA samples extracted from clinical specimens. No cross-amplification was observed within these waterfowl species or with other well-known avian mycoplasmas. The average sensitivity of the assays was 10^1–10^2 genomic equivalents per reaction. These conventional PCR assays can be run simultaneously in the same PCR conditions, and the species can be differentiated directly (without sequence

analysis) by gel electrophoresis due to the different sizes of the amplicons. The species-specific PCR assays were found to be suitable for routine use at regular veterinary diagnostic laboratories and promote the rapid, simple and cost-effective differentiation of these waterfowl *Mycoplasma* species.

Multilocus sequence typing (MLST) provides a valuable insight into the phylogeny of bacterial pathogens; however, there was no such assay available for *M. anserisalpingitidis*. A novel MLST scheme was developed to analyse phylogenetic relationships between *M. anserisalpingitidis* samples originating from different geographical locations (Hungary, Poland, Ukraine, China and Vietnam). Five loci (*atpG, fusA, pgiB, plsY,* and *uvrA*) were selected for the final MLST study. The examined 89 *M. anserisalpingitidis* samples yielded 76 unique sequence types (STs) with a 0.994 Simpson's index of diversity. The created phylogenetic tree sorted the samples into three distinct clades and several subclades. Generally, samples originating from the same geographical locations or livestick integration clustered together. Some isolates showed close relationships to the *M. anatis* outgroup due to a gene sequence similarity. The method proved to be a valuable and cost-effective tool for sequence typing of this waterfowl *Mycoplasma* species, enabling a better understanding of its phylogeny.

To summarise this work, new results and novel techniques were demonstrated in order to study the most common waterfowl *Mycoplasma* species. Detailed antibiotic susceptibility profiles of Hungarian *M. anserisalpingitidis* field isolates were defined for the first time, demonstrating elevated MIC values for several antibiotics. Complete genomes of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* strains/isolates were created for the first time to support novel molecular biology researches. In order to facilitate the diagnosis of these *Mycoplasma* species, specific PCR assays were designed and evaluated for the first time. Finally, a fast and cost-effective genotyping method was designed for the first time for *M. anserisalpingitidis* to analyse its phylogeny.

Összefoglalás

A *Mycoplasma anserisalpingitidis*, *M. anatis*, *M. anseris* és *M. cloacale* ludakat és kacsákat fertőző *Mycoplasma* fajok. Az általuk okozott betegségekre a kloáka és a nemi szervek gyulladása, csökkent tojástermelés, megnövekedett embrióelhalás, esetenként légzőszervi és idegrendszeri tünetek a legjellemzőbbek. Magyarországon a ludak *M. anserisalpingitidis* okozta megbetegedése jelentős gazdasági károkat okoz.

A vízibaromfi patogén *Mycoplasma* fajok ellen kereskedelmi forgalomban nem kapható vakcina, így a megfelelő tartási körülményekkel és célzott antibiotikum terápiával lehet védekezni e kórokozókkal szemben. Vizsgálataink során *in vitro* meghatároztuk 38 *M. anserisalpingitidis* klinikai izolátum érzékenységét tizenhárom antibiotikummal és egy antibiotikum kombinációval szemben. A baktérium törzseket (egy minta kivételével) lúdból izoláltuk, amelyeket 2011 és 2015 között Magyarország számos településéről gyűjtöttünk. Az izolátumok jelentős része magas minimális gátló koncentráció (MIC) értéket mutatott tilmikozinnal, oxitetraciklinnel, norfloxacinnal és difloxacinnal szemben. Vizsgálatainkban a valnemulin, a tiamulin és a tilvalozin bizonyultak a leghatásosabb antibiotikumoknak, azonban minden alkalmazott antibiotikummal szemben találtunk olyan izolátumokat, amelyek magas MIC értékeket mutattak. Ezen eredmények felhívják a figyelmet az antibiotikum érzékenység meghatározásának a fontosságára a kezelés megkezdése előtt.

A korszerű molekuláris biológiai vizsgálatok megkövetelik, hogy ismerjük a vizsgált kórokozó genetikai állományát, azonban vizsgálataink előtt egyik *Mycoplasma* fajnak sem volt elérhető a teljes genom szekvenciája. *De novo* teljes genom szekvenálás során meghatároztuk a *M. anserisalpingitidis* típus törzsnek (ATCC BAA-2147) és két klinikai izolátumnak, valamint a *M. anatis*, *M. anseris* és *M. cloacale* típus törzseinek (NCTC 10156, ATCC 49234 és NCTC 10199) bázis sorrendjét. A teljes genomokat elhelyeztük a GenBank-ban, hogy segítsük további tudományos munkák születését.

A vízibaromfi patogén *Mycoplasma* fajok gyakran együtt fordulnak elő a gazdaállatokban, azonban a faj szintű azonosításuk egy hagyományos mikrobiológiai laboratórium számára nehéz, mivel ezek a törzsek hasonló morfológiai, tenyésztési és biokémiai tulajdonságokkal bírnak. A faj szintjén történő megkülönböztetésük korábban egy nemzetség-specifikus polimeráz láncreakció (PCR)-termék megszekvenálásával történt. E probléma megoldására háztartási génekre faj-specifikus PCR rendszereket terveztünk, amelyekkel pontosan beazonosíthatjuk a vizsgált mycoplasmákat. A tervezett PCR vizsgálatok egyedileg azonosítják a *M. anserisalpingitidis*, *M. anatis*, *M. anseris* és *M. cloacale* fajokat, akár közvetlenül a klinikai mintából kivont DNS-ből is. Vizsgálataink során nem tapasztaltunk keresztreakciót egyéb madár *Mycoplasma* fajokkal, a tesztek átlagos érzékenysége 10^1−10^2 DNS templát volt. Ezek a konvencionális PCR tesztek azonos

hőprofilon egyidőben futtathatók, és a fajok gélelektroforézist követően a termékméret alapján elkülöníthetők, így nincs szükség szekvenálásra. A faj-specifikus rendszerek eredményesen alkalmazhatóak a rutin állatorvosi laboratóriumi diagnosztikai vizsgálatok során, ezzel lehetővé teszik a vízibaromfi patogén *Mycoplasma* fajok gyors, egyszerű és költséghatékony elkülönítését.

A multilókusz szekvencia tipizálás (MLST) módszerével patogén baktériumok leszármazástani (filogenetikai) vizsgálatát lehet elvégezni, azonban ilyen módszer a *M. anserisalpingitidis*-re korábban még nem állt rendelkezésre. Az MLST séma kidolgozásával lehetőség nyílt a leszármazástani kapcsolatok vizsgálatára különböző eredetű (magyar, lengyel, ukrán, kínai és vietnámi) *M. anserisalpingitidis* minták között. A végső MLST vizsgálathoz öt lókuszt (*atpG, fusA, pgiB, plsY, uvrA*) választottunk ki. A vizsgálatba bevont 89 *M. anserisalpingitidis* mintánál 76 egyedi szekvencia típust (ST) különböztettünk meg, 0,994 Simpson-féle diverzitási index értékkel. A létrehozott leszármazási fa 3 elkülönülő csoportba és számos alcsoportba sorolta a mintákat. Általánosságban elmondható, hogy az azonos földrajzi helyről, vagy ugyanazon integrációból származó minták közös csoportot alkottak. Számos klinikai izolátum szoros kapcsolatot mutatott a külső referenciaként (outgroup) használt *M. anatis* mintákkal. A módszer értékes és költséghatékony eszköznek bizonyult a vizsgált *Mycoplasma* faj szekvencia típusainak meghatározásában, amely hasznos lehet a járványtani nyomozáshoz.

Összefoglalva, új eredményeket és technikákat mutattunk be a leggyakoribb, vízibaromfikat fertőző *Mycoplasma* fajok tanulmányozására. Elsőként állapítottuk meg hazai *M. anserisalpingitidis* törzsek antibiotikum érzékenységét, amely magas MIC értékeket fedett fel számos antibiotikummal szemben. Elsőként hoztuk létre *M. anserisalpingitidis*, *M. anatis*, *M. anseris* és *M. cloacale* törzsek teljes genom szekvenciáit, támogatva új molekuláris biológiai kutatások létrejöttét. Annak érdekében, hogy elősegítsük ezen *Mycoplasma* fajok diagnosztikai vizsgálatát, elsőként terveztünk megbízható PCR rendszereket a kimutatásukra. Legvégül, elsőként fejlesztettünk egy gyors és költséghatékony genotipizáló módszert *M. anserisalpingitidis* minták filogenetikai vizsgálatára.

2. Introduction

2.1. History

Mycoplasma anserisalpingitidis, *M. anatis*, *M. anseris* and *M. cloacale* colonise geese and ducks. They can cause significant losses to the waterfowl industry (Stipkovits and Szathmary, 2012).

The chronic respiratory disease in ducks was first described in 1955 when the association between the pathological changes and a novel *Mycoplasma* species was indicated (Fahey, 1955; Roberts, 1964). *Mycoplasma* infection of geese was first reported in 1970 (Kosovac and Djurisic, 1970). Since then, several *Mycoplasma* and *Acholeplasma* (a *Mycoplasma*-related genus) species were described from geese and ducks.

M. anserisalpingitidis was first isolated from the phallus lymph of a goose with phallus inflammation in 1983 by László Stipkovits' team in Hungary (the strain numbered 1220), along with other mycoplasma isolates. However, description of the new species and the officially proposed name was published only in 2020 (Stipkovits et al., 1984b; Varga et al., 1986; Volokhov et al., 2020). The organism was referred to as *Mycoplasma* sp. 1220 or *M. anserisalpingitis* in publications between 1983 and 2020. Another strain (strain 1219) was described a few years later of its isolation and was named as *M. anseris* (Stipkovits et al., 1984b; Bradbury et al., 1988).

M. anatis was first described in 1964, in the United Kingdom. The author reported a sinusitis outbreak, and a co-infection of influenza A and a *Mycoplasma* species (Roberts, 1964).

Although *M. cloacale* isolation was first reported from a turkey (Bradbury and Forrest, 1984), this species commonly occurs with other *Mycoplasma* species in waterfowl (Stipkovits et al., 1984b, 1986; Varga et al., 1986; Varga, 1993; Carnaccini et al., 2016; Gyuranecz et al., 2020).

The literature concerning the epidemiology or pathogenesis of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* is lacking. Many of the available papers are case reports, most of which are Hungarian studies. As more than one *Mycoplasma* species were described in the majority of the studies, the exact pathogenic features of these agents are not defined. Among these four species, *M. cloacale* is considered as a commensal *Mycoplasma* (Bradbury et al., 1987; Stipkovits and Kempf, 1996; Stipkovits and Szathmary, 2012); however, it was also reported that this species alone can cause clinical signs in geese (Stipkovits et al., 1986; Bradbury et al., 1987). Thus, the unique identification is important for differentiation this bacterium from other pathogen *Mycoplasma* species.

2.2. Aetiology

Mycoplasma species belong to the family *Mycoplasmataceae* in the order *Mycoplasmatales* and the class *Mollicutes* (Garrity et al., 2004). The members of the genus *Mycoplasma* are characterised by the 16S rRNA gene sequences, serologic tests and phenotypic characteristics (Kleven, 2008).

Mycoplasmas are Gram-negative, small prokaryotes that lack a cell wall and are bounded by a triple-layered plasma membrane, resulting in pleomorphic shapes. The cell size of mycoplasmas is about 0.2-0.8 μm in diameter (Kleven, 2008; Brown et al., 2018). Based on the available data, the cell size of *M. anserisalpingitidis* is 600-850 nm (Volokhov et al., 2020), and the average size for *M. cloacale* is 515 nm (Bradbury and Forrest, 1984).

Mycoplasma species are characterised by a small genome (580,000 – 1,350,000 bp) with G+C content of 23.7–44.0%. Certain species have fewer than 300 genes (Stipkovits and Kempf, 1996; Sirand-Pugnet et al., 2007; Kleven, 2008). The UGA codon stands for a universal stop codon; however, most of the species in the class *Mollicutes* use it as a tryptophan coding sequence (Sirand-Pugnet et al., 2007).

It was proposed to use the 16S rRNA similarity value to indicate separate, genetically distant species. If two strains of the same cluster or group have 16S rRNA sequence similarity above 97%, they are most likely to belong to the same species (Pettersson et al., 2000; Volokhov et al., 2012). Nevertheless, several closely related *Mycoplasma* species with 16S rRNA gene similarity greater than 97% demonstrated serological, genetic and ecological features that allowed defining them as distinct, individual species. High similarity of the 16S rRNA genes was observed in *M. anserisalpingitidis* with *M. anatis*, and *M. anseris* with *M. cloacale,* too, similarity percentage was 98.3-99.0%. Three-target sequence analysis, which uses the 16S rRNA, 16S-23S rRNA intergenic transcribed spacer region (ITS), and DNA-directed RNA polymerase subunit beta (*rpoB*) genes demonstrated to be a reliable and useful taxonomic tool for the species differentiation within the family *Mycoplasmataceae*. According to the phylogenetic tree of *Mycoplasma* species and sequence analysis, *M. anserisalpingitidis* is closely related to *M. anatis* and they belong to the Synoviae cluster, whilst the Hominis cluster included *M. anseris* and *M. cloacale* and these species are also in a close phylogenetic relationship (Volokhov et al., 2012, 2020).

All four *Mycoplasma* species could be a part of the normal microflora of domestic geese and ducks, carried without any signs of *Mycoplasma* infection (i.e. unapparent infections) (Bencina et al., 1987; Hinz et al., 1994; Stipkovits and Szathmary, 2012). However, the high isolation rate from diseased animals and experimental studies clearly confirm the pathogenic role of these bacteria.

Besides the most common waterfowl *Mycoplasma* species, sporadic identifications were reported for *M. columbinasale*, *M. gallinaceum* (Tiong, 1990), *M. gallinarum* (Stipkovits et al., 1975), *M. gallisepticum* (Jordan and Amin, 1980; Bencina et al., 1988; Levisohn and Kleven, 2000), *M. glycophilum*, *M. imitans*, *M. lipofaciens* (Stipkovits and Kempf, 1996), *M. synoviae* (Bencina et al., 1988), a novel *Mycoplasma* species (Carnaccini et al., 2016) and a few acholeplasmas (Stipkovits and Kempf, 1996) in domestic ducks and geese.

2.3. Host specificity and geographical distribution

Mycoplasmas tend to be host-specific, but much data demonstrate that these bacteria are not always restricted to a single animal species (Bencina et al., 1987; Kleven, 2008; Stipkovits and Szathmary, 2012). Moreover, the co-occurrence of several waterfowl mycoplasmas have been described even in the same animal (Stipkovits et al., 1984b; Bradbury et al., 1988; Varga et al., 1986; Behr et al., 1990; Varga, 1993; Stipkovits and Szathmary, 2012).

M. anserisalpingitidis is a pathogen of domestic geese (*Anser anser*) (Stipkovits and Szathmary, 2012) and it was recently identified in swan geese (*Anser cygnoides*) (Gyuranecz et al., 2020). *M. anatis* colonises mainly domestic ducks (*Anas platyrhynchos*) (Stipkovits and Szathmary, 2012), but this organism was also described in several wild duck hosts (black duck, canvasback, mallard, teal, and shoveler) and other type of birds (coot) (Poveda et al., 1990; Goldberg et al., 1995), and in healthy geese as well (Bencina et al., 1987). *M. anseris* was only described in domestic geese and no data suggests that this species colonises other animals (Stipkovits and Szathmary, 2012). *M. cloacale* could be isolate from various birds; however, the frequent occurrence of this species in domestic and wild ducks, along with domestic geese suggests that members of the order *Anseriformes* may be more common hosts for *M. cloacale* than other birds (Bradbury et al., 1987; Hinz et al., 1994; Goldberg et al., 1995; Stipkovits and Szathmary, 2012; Gyuranecz et al., 2020).

M. anserisalpingitidis and *M. anatis* may have separated from a common ancestor approximately 20–40 million years ago. Similarly, geese and ducks most likely derived from their common avian ancestor approximately 20.8 million years ago. Therefore, it was speculated that the evolution of these two, closely related *Mycoplasma* species might have been in direct association with the evolution of their natural hosts (Lu et al., 2015; Volokhov et al., 2020).

Although the majority of the available publications are Hungarian studies, diseases of domestic geese associated with *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* alone or in co-infections were also detected in other European countries and other parts of the World. These bacteria were described in Europe (Germany, France (Stipkovits et al., 1986), Czech Republic (Stipkovits et al., 1986; Stipkovits and Kempf, 1996), Poland (Stipkovits et al., 1986),

former Soviet Union, Russian Federation and Ukraine (Stipkovits et al., 1986; Sprygin et al., 2012)). In the USA, *M. anseris* and *M. cloacale* was recognised in domestic geese, showing clinical signs (Carnaccini et al., 2016). In China, the presence of *M. anseris* was reported before (Stipkovits and Kempf, 1996), and *M. anserisalpingitidis* and *M. cloacale* have been recently described in swan geese showing signs of the purulent or necrotic cloaca and phallus inflammation (Gyuranecz et al., 2020).

Diseases of domestic ducks connected with *Mycoplasma* infection was reported in Hungary (Ivanics et al., 1988), in the United Kingdom (Roberts, 1964; Jordan and Amin, 1980), and Singapore (Tiong, 1990). Lesions associated with the presence of *Mycoplasma* species were investigated in wild ducks in the USA (Goldberg et al., 1995).

Moreover, the concerned *Mycoplasma* species were identified from symptomless domestic and wild waterfowl in the former Yugoslavia (Bencina et al., 1987), Germany (Hinz et al., 1994), France (Bradbury et al., 1987), Spain (Poveda et al., 1990), United Kingdom (Bradbury et al., 1987) and Egypt (Goldberg et al., 1995).

2.4. Epidemiology and pathogenesis

In waterfowl, co-infection of *Mycoplasma* species with other bacterial or viral pathogens may lead to more severe disease manifestations and their consequences. *Pasteurella multocida* was isolated along with *M. anseris* and *M. cloacale* in ganders with reproductive diseases (Carnaccini et al., 2016). Co-occurrence with *P. multocida* in ducks was also observed (Tiong, 1990). *M. anatis* and *M. gallisepticum* together were recovered from birds with respiratory diseases (Jordan and Amin, 1980), and the influenza A virus clearly worsened the mycoplasmal infection in ducks (Roberts, 1964).

Horizontal transmission of *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* was hypothesized in a previous experiment (Stipkovits et al., 1986), as the pathogens were detected from the healthy ganders which were introduced in an affected flock after eliminating birds showing cloaca and phallus inflammation. Since *M. cloacale* can be isolated from the cloaca, it was hypothesized that this species may spread by faecal contamination between wild and domestic birds (Bradbury et al., 1987). Based on the observations and experimental studies, these waterfowl *Mycoplasma* species have the potential to transmit vertically as well (Stipkovits et al., 1984a, 1984c; Samuel et al., 1995; Stipkovits and Kempf, 1996; Dobos-Kovács et al., 2009).

There is no detailed study about the pathogenesis of *Mycoplasma* infections in waterfowl, but it is clear that avian *Mycoplasma* species require the attachment to host cell surfaces. Moreover, certain species are known to have the ability to penetrate cells (Kleven, 2008). The bacterial sialidase (also called neuraminidase) activity is associated with the

creation of binding sites on the host's epithelial cells leading to invasion (Corfield, 1992). The reptile pathogen *M. alligatoris* has sialidase activity, and this species showed rapid invasiveness and necrotizing effects compared to its attenuated sibling species, *M. crocodyli* which lacks this enzyme (Brown et al., 2004; Hunt and Brown, 2005). The poultry pathogens *M. synoviae* and *M. gallisepticum* bind to the host cell via receptors containing sialic acid residues (Razin and Barile, 1985; Berčič et al., 2008), and the intensive neuraminidase enzymatic activity (NEAC) of the strains can be associated with a higher invasiveness and virulence (Berčič et al., 2008). The type strain of *M. anseris* was isolated from a goose showing clinical signs, and that strain showed a moderate NEAC. The *M. cloacale* type strain had a weak NEAC; nevertheless, the pathogenicity of this strain is unknown. The type strain of *M. anatis* was associated with respiratory diseases; however, its NEAC was negative (Roberts, 1964; Bradbury and Forrest, 1984; Bradbury et al., 1988; Berčič et al., 2008).

2.5. Clinical signs

During infection with *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* alone or in combinations, inflammation of the cloaca and genital tract are the main symptoms in ganders (Figure 1). The mucosal membrane of the phallus becomes red, oedematous, later necrotic and finally, the phallus can slough. The severe phallic lesions compromise the functionality of the organ, thus preventing mating, which results in high infertility rates. Fibrinous material can congest the cloaca, and the congested uric acid can cause a severe visceral gout (Stipkovits et al., 1986; Bradbury et al., 1988; Behr et al., 1990; Stipkovits and Kempf, 1996; Carnaccini et al., 2016). *M. anserisalpingitidis*- and *M. anseris*-infected layers can have conjunctivitis with lacrimation, the birds show signs of depression, the intake of drinking water increases and many birds can die (Dobos-Kovács et al., 2009). Moreover, a decreased egg production, increased number of infertile eggs, embryo mortality and abnormally looking eggs are associated with the presence of these *Mycoplasma* species (Stipkovits et al., 1986; Stipkovits and Kempf, 1996; Dobos-Kovács et al., 2009). Nasal discharge, coughing, difficult breathing, lacrimation, conjunctivitis, increased morbidity and mortality are the signs of *M. anserisalpingitidis* and *M. anseris* infection in goslings. The young birds move with difficulty, and prefer sitting, leg weakness, diarrhoea, loss of body weight and weakness can be observed. Nervous signs, such as tremor and torticollis were also seen occasionally (Stipkovits et al., 1993; Stipkovits and Kempf, 1996).

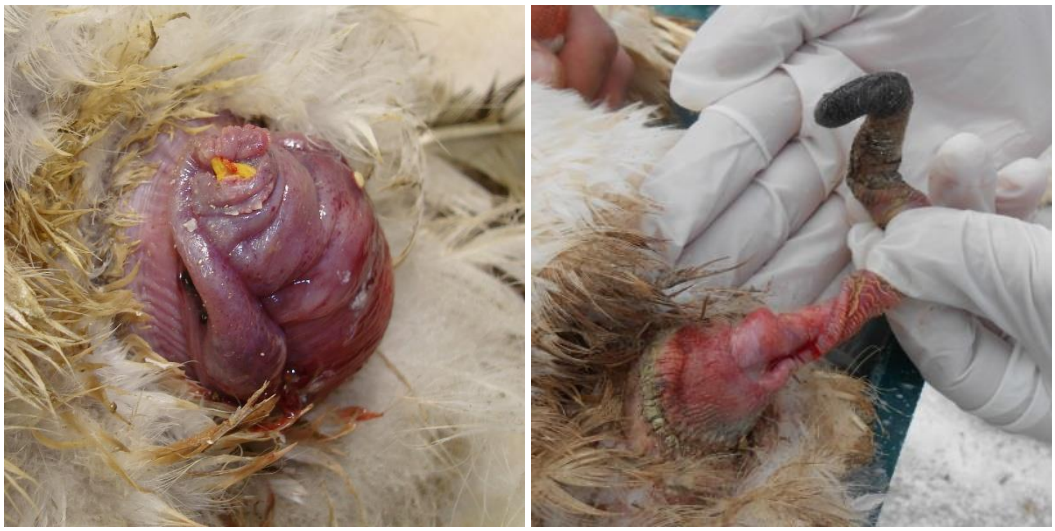

Figure 1. Phallus inflammation (photo kindly provided by Ákos Thuma) and necrotized phallus (photo taken by Miklós Gyuranecz).

Layers were infected with *M. anseris* and *M. cloacale* together in an experimental study. The rate of the infertile eggs significantly increased 1 month after the infection, and the egg production decreased after the 2nd month. The bodyweight of the infected laying geese significantly decreased. The freshly-hatched goslings of the infected layers showed a significantly lower weight and decreased growth rate compared to the control group (Stipkovits et al., 1984a, 1984c; Dobos-Kovács et al., 2009). An *in ovo M. anserisalpingitidis* infection resulted in significantly shorter mean body length, and the embryo mortality significantly increased. Goslings hatched from infected embryos weighed less and had higher mortality. Rhinitis was observed after experimental infection of post-hatched goslings (Stipkovits et al., 1987).

The clinical signs of *M. anatis* infection included weakness of the legs, preferred sitting, along with dyspnoea and diarrhoea with watery and greenish faeces in ducklings. Some birds showed nervous symptoms, such as disturbances of the equilibrium, retrograde movement, torticollis and twisting of the neck. Fatal cases were observed as well (Ivanics et al., 1988). Moreover, an association was hypothesized between *M. anatis* infection and mortality in wild ducks (Goldberg et al., 1995).

The pipping success in *M. anatis*-inoculated eggs was significantly lower, and the decrease in the hatching success was more pronounced. The mass of the hatchlings was lower, and only half of them survived to 24 hours (Samuel et al., 1995). Inoculating duck embryos with *M. anatis* also increased their mortality (Stipkovits, 1979).

2.6. Pathology

The phallus lesions of ganders associated with *M. anserisalpingitidis*, *M. anseris* and *M. cloacale* infection were characterised by sero-fibrinous inflammation of the lymph sinus'

mucous membrane and the glandular part of the organ, and it can also get necrotic (Figure 2 A). The presence of the severe inflammatory reactions in ducts limits the normal flow of the semen and lymph. This may also explain the atrophy and degenerative changes of the phallus. The formation of a stagnant environment and the impossibility to purge the bacteria and necrotic debris aggravates the inflammatory reaction. Bacteria from the cloacal region can take advantage of the stagnant environment and start to proliferate. Atrophy of the testicles can also be observed (Stipkovits et al., 1986; Stipkovits and Kempf, 1996; Carnaccini et al., 2016). In certain cases, the outer reproductive organs do not show pathologic changes, but the abdominal ones are affected in seemingly healthy birds. Airsacculitis and peritonitis are also common signs in the infected ganders (Stipkovits et al., 1986; Stipkovits and Kempf, 1996). Airsacculitis, peritonitis and pericarditis were observed in *Mycoplasma*-infected laying geese. Exudates and infiltrations with immune cells were seen in many parts of the reproductive organs, accompanied mainly with salpingitis and degenerated follicles (Figure 2 B). General immune cell infiltration was noted around the portal blood vessels in the liver (Stipkovits et al., 1984a, 1984c; Dobos-Kovács et al., 2009). Immune cell and/or fibrin infiltration was observed in the epicardium, pericardium, peritoneum, air sacs, and lungs in goslings suffering from *M. anserisalpingitidis* and *M. anseris* infection. Their lungs contained large quantity of blood and were oedematous, and serous mucus was seen in the lumen of the trachea and the sinus. The joints were swollen in some cases. Similar infiltrations were seen in the cerebral membranes and chambers of young birds showing nervous symptoms (Stipkovits et al., 1993; Stipkovits and Kempf, 1996).

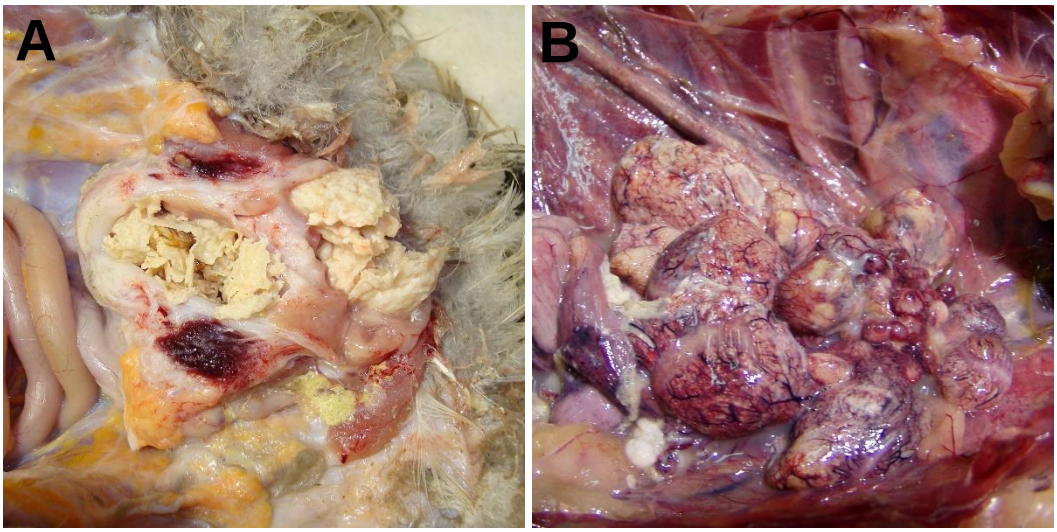

Figure 2. A: sero-fibrinous phallus inflammation. B: peritonitis and salpingitis with degenerated follicles (photos kindly provided by Ákos Thuma).

Laying geese experimentally infected with *M. anseris* and *M. cloacale* had peritonitis and airsacculitis, and the attachment of yolk follicles to the peritoneum was seen. In the

goslings of these infected birds, airsacculitis, pneumonia and omphalitis were observed (Stipkovits et al., 1984a, 1984c; Dobos-Kovács et al., 2009). As a consequence of the *M. anserisalpingitidis in ovo* infection, pathological lesions were seen in the chorioallantoic membrane, and the embryos' liver and respiratory tract. The goslings hatched from infected eggs, and pathological examinations revealed cellular infiltrations in the pleura, lung, large air passages and nasal conchae. The follicles of the bursa of Fabricius and the thymic cortex were atrophied. Post-hatch infiltration resulted in enhanced secretory activity, vacuolar degeneration and cellular infiltration of the nasal tissues. Similar infiltration occurred in the pleura, in the pulmonary tissues and the large air passages (Stipkovits et al., 1987).

The air sacs and pericardium were covered with moderate amounts of fibrin, and peritonitis was observed in ducks suffering from *M. anatis* infection (Stipkovits, 1979; Ivanics et al., 1988). The lungs were hyperaemic and slightly oedematous, the mucosa of the nasal turbinates was slightly hyperaemic, swollen and covered with catarrhal mucus. The mucosa of the empty intestinal tract and the liver were hyperaemic and swollen. Fibrinous salpingitis was observed as well. The leptomeninx was thickened and infiltrated by immune cells in the brain stem, cerebral hemispheres and cerebellum of the birds showing nervous signs (Ivanics et al., 1988).

During experimental infection of ducks with *M. anatis* (inoculated intranasally, into the infundibulum, into the airsac and intracranially), lesions were found in the lungs, parietal and visceral pleura, and tracheal and turbinate mucosa in all groups. Moreover, an infiltration of immune cells was observed in the above mentioned brain regions and all these observations were similar to the ones of the naturally infected ducks (Ivanics et al., 1988).

2.7. Diagnosis

Based on the available literature, the waterfowl *Mycoplasma* species were isolated from multiple organs of the animals, like the brain, respiratory tract, peritoneum, liver, reproductive organs, cloaca or embryos (Stipkovits, 1979; Jordan and Amin, 1980; Stipkovits et al., 1986, 1993; Bencina et al., 1988; Ivanics et al., 1988; Hinz et al., 1994; Goldberg et al., 1995; Stipkovits and Kempf, 1996; Dobos-Kovács et al., 2009). This emphasizes the multi-organotropic nature of these species. The routinely collected samples from live animals are cloacal and tracheal swabs, and phallus lymph and sperm from males. These samples are appropriate for bacterial culturing and/or molecular identification.

The four waterfowl *Mycoplasma* species require a protein-rich growth medium containing serum and cholesterol, and are inhibited by digitonin. *M. anserisalpingitidis* and *M. anatis* are phosphatase-positive glucose-utilizers, whilst *M. anseris* and *M. cloacale* are phosphatase-negative arginine-hydrolysers; therefore, these species pairs have

undistinguishable biochemical properties (Stipkovits et al., 1984b; Kleven, 2006, 2008). Mycoplasmas develop only faint or no turbidity in broth cultures; consequently, alternative methods are used to detect mycoplasmal growth. These include the incorporation of substrates (such as glucose and arginine) which are fermented or hydrolysed by the mycoplasmas causing acidification or alkalisation of the medium (Hannan, 2000). The optimum growth is at 37 °C, which is supported by 5% CO_2. All four species form “fried-egg” colonies on solid media (Stipkovits et al., 1984b; Kleven, 2008) (Figure 3). Based on our team’s observations, all these species form colonies after 1-4 days. Therefore, the characterisation of the phenotypic features of these mycoplasmas is not sufficient for their routine identification to the species level (Stipkovits et al., 1984b; Sprygin et al., 2012).

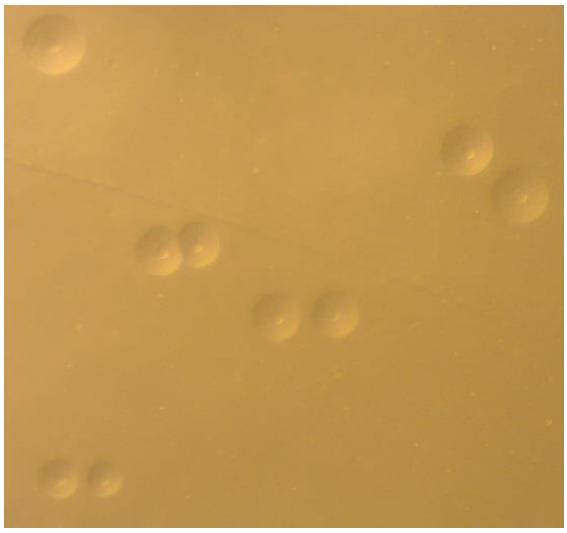

Figure 3. *M. anserisalpingitidis* colonies on solid media (x30 magnification).

Practically, these species can be identified using serological methods, such as growth inhibition test and immunofluorescent antibody test; however, these tests are time-consuming, require a pure culture of isolates and qualified reference antisera, which are not available at routine veterinary laboratories in most countries either due to absence of national veterinary mycoplasma reference centres or due to the limited use of animals for the production of antibody reagents. Moreover, closely related species, such as *M. anserisalpingitidis* and *M. anatis* may cross-react in conventional serological tests (Stipkovits et al., 1984b; Volokhov et al., 2020).

The identification of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* to the species level is possible with a *Mycoplasma* genus-specific PCR assay, which targets the 16S-23S rRNA ITS (Lauerman et al., 1995); however, the species identification requires the direct sequencing of the obtained PCR amplicons. This is not always feasible at routine veterinary laboratories, and requires additional reagents and skills. Moreover, this kind of identification is practically impossible for PCR products amplified from the birds co-infected with multiple

Mycoplasma species. To the best of our knowledge, no *M. anatis*-, *M. anseris*- or *M. cloacale*-specific PCR assays have been published, and only one study on the species-specific PCR detection and phylogenetic identification of *M. anserisalpingitidis* isolates in geese from the Russian Federation and Ukraine was published (Sprygin et al., 2012). A core genome MLST scheme (cgMLST) was designed for *M. anserisalpingitidis* by our research group (Kovács et al., 2020) which was the first published genotyping assay to differentiate between strains of this *Mycoplasma* species. That method requires pure bacterial strains, a whole-genome sequencing and high-quality input data.

2.8. Treatment and control

Clinically manifested mycoplasmosis can occur in waterfowl under excessive stress. Inadequate housing, such as the high density of birds, limited space and water supply and mixed-aged fowls increase the anxiety of the birds; moreover, sexual activity of the animals, extensive egg production and transport of the birds are stress factors as well (Stipkovits et al., 1986; Ivanics et al., 1988; Stipkovits and Kempf, 1996). The screening for *Mycoplasma* species of the fowls is crucial to control mycoplasmosis. Diseases can be reduced with adequate housing and better hygienic conditions.

A combined vaccine against *M. anserisalpingitidis* and *M. anseris* was developed and assessed for efficacy, and a registration dossier was submitted under the name Anservac, in Hungary (Földművelésügyi és Vidékfejlesztési Értesítő, 2006; Volokhov et al., 2020). Moreover, an American patent application publication can be found associated with waterfowl *Mycoplasma* species (Szathmary and Stipkovits, 2016). Despite there is no commercially available vaccine against these mycoplasmas. Therefore, ‘tailor-made’ autovaccine therapies are occasionally used in diseased waterfowls.

Since there is no commercial vaccine against *M. anserisalpingitidis*, *M. anatis*, *M. anseris* or *M. cloacale*, an antibiotic treatment can be applied for the control of the *Mycoplasma* infections. Mycoplasmas are resistant to β-lactam antimicrobials because of the lack of cell-wall and the bacteria are also resistant to membrane synthesis inhibitors (Stipkovits and Kempf, 1996; Hannan, 2000). Antibiotics such as quinolones, tetracyclines, macrolides and pleuromutilins which induce DNA fragmentation or inhibition at the level of protein synthesis are the drugs of choice for the therapy of mycoplasmosis. Among the macrolides, erythromycin showed high effectiveness against *Mycoplasma* strains which ferment glucose, while arginine-hydrolysing strains proved to be less susceptible to this compound. However, there are big variations in antibiotic sensitivity among strains of one species (Stipkovits and Kempf, 1996).

Based on the available literature, the *in vitro* antibiotic susceptibility of these four *Mycoplasma* species were published in only one paper (Stipkovits and Szathmary, 2012). The authors described MIC_{50} (lowest concentration that inhibited the growth of 50% of the investigated strains), MIC_{90} (lowest concentration that inhibited the growth of 90% of the investigated strains) and average MIC values against seven antibiotics; however, detailed data were not shared concerning the examination method or the examined isolates. High MIC values were published for chlortetracycline, oxytetracycline, lincomycin, and tylosin, whilst tiamulin was found to be the most effective agent out of the tested compounds.

Literature concerning the medication of mycoplasmosis in geese and ducks is scarce as well. *M. anserisalpingitidis* and *M. cloacale* infected geese were treated with lincomycin-spectinomycin combination, tylosin and tiamulin. The drugs were applied either alone or in combinations. The number of geese with inflamed phalluses and the infertility rate of eggs decreased; moreover, the hatching rate and egg production increased as the consequence of the treatments. The data suggested the beneficial effects of these drugs against mycoplasmosis (Czifra et al., 1986; Varga et al., 1986; Varga, 1993). Tylosin and chlortetracycline treatment improved the fertility of a geese flock suffering from reproductive diseases caused by *M. anseris*, *M. cloacale*, an unknown *Mycoplasma* species and *Pasteurella*. However, the birds never returned to the initial production rates because of the irreversible deformities to the male genitalia (Carnaccini et al., 2016).

3. Aims of the study

The aims of the study were:

Ad 1. to determine the *in vitro* susceptibility of Hungarian *M. anserisalpingitidis* field isolates to thirteen different antibiotics and an antibiotic combination.

Ad 2. to sequence *de novo* the complete genomes of three isolates (type strain and two field isolates) of *M. anserisalpingitidis*, and the type strains of *M. anatis*, *M. anseris*, and *M. cloacale*.

Ad 3. to develop an effective and robust molecular test for the identification of *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale* in avian clinical specimens.

Ad 4. to develop a reproducible and useful MLST assay that can be used to analyse the phylogenetic relationships between *M. anserisalpingitidis* strains.

4. Materials and methods

4.1. Samples and isolation

4.1.1. Samples

To determine the antibiotic susceptibility of *M. anserisalpingitidis* isolates, the type strain (ATCC BAA-2147) and 38 field isolates from geese and a duck originating from different parts of Hungary were tested. The field isolates were collected between 2011 and 2015, from phallus tissue and phallus lymph, cloaca, follicle and respiratory tract samples (Table 1, Figure 4).

For the *de novo* sequencing, the type strains of *M. anserisalpingitidis* (ATCC BAA-2147), *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234), and *M. cloacale* (NCTC 10199) were purchased directly from the repositories. In addition, two *M. anserisalpingitidis* field isolates (MYCAV 93 and 177) were also included in the study. Data of the concerned strains/isolates are showen in Tables 1-4 (Figure 4).

To evaluate the species-specific PCR assays, 18 *M. anserisalpingitidis*, eight *M. anatis*, 13 *M. anseris*, and 18 *M. cloacale* field isolates were investigated, obtained from domestic geese and ducks, wild ducks and a chicken (Tables 1-4, Figure 4). Furthermore, 28 clinical specimens, including cloacal swabs, follicle tissue, trachea tissue, phallus lymph, and semen were also examined in this study (Table 19, Figure 4). The tested *Mycoplasma* field isolates and the avian clinical specimens were collected mainly in Hungary, between 2003 and 2018. Two North American samples were also included.

To develop a novel MLST scheme, 82 *M. anserisalpingitidis* isolates, including the ATCC BAA-2147 type strain, were recovered from domestic geese, swan geese and a domestic duck between 1983-2019 originating from Hungary (n=71), Poland (n=8), China (n=2) and Vietnam (n=1). The isolates originated from the cloaca, phallus and phallus lymph, trachea, follicle, semen, and lung and air sac of the animals. Among these *M. anserisalpingitidis* samples, 36 field isolates were collected from flocks of a Hungarian livestock integration which were sampled frequently between 2011-2018. The distance between the sampled farms of the integration ranged from 12 to 230 km. Moreover, the field isolates originating from the same flock or same animal's different organs were also indicated in the study (Table 1, Figure 4). The *M. anatis* type strain (NCTC 10156) and six *M. anatis* field isolates recovered from domestic geese and ducks between 2011-2014 from Hungary (Table 2, Figure 4) were examined in the study as an outgroup. Also, the MLST study included seven clinical specimens (transport medium or tissue sample without cultivation) containing *M. anserisalpingitidis* DNA, originating from Poland (n=6) and Ukraine (n=1) (Table 1, Figure 4).

All the samples were collected in routine diagnostic examinations or necropsies by our research group and our collaborators, thus ethical approval was not required for any of the studies.

For all studies, DNA was extracted from *Mycoplasma* strains, field isolates or clinical specimens using the QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions for Gram-negative bacteria. The performed examinations on each strain/isolate and clinical specimen are marked in Tables 1-4.

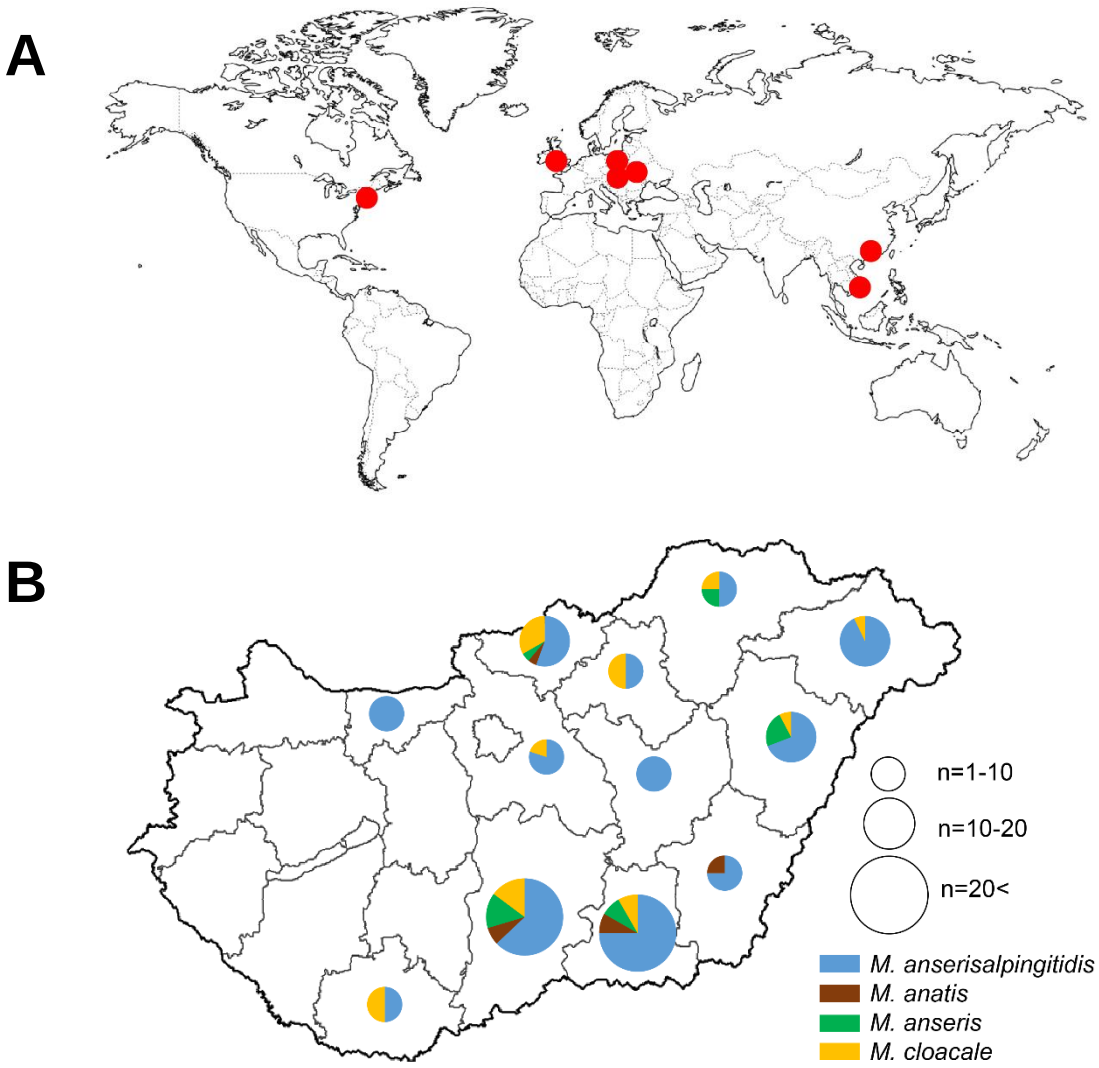

Figure 4. A: Map showing the geographical origin of the waterfowl *Mycoplasma* type strains, field isolates and clinical specimens analysed in the studies.
B[a]: Map of Hungary showing the geographical origin of the waterfowl *Mycoplasma* field isolates examined in the studies.
[a]The size of the pie charts represents the number (n) of isolates. Colours indicate the *Mycoplasma* species. Field isolates with unknown places (MYCAV 344, MYCAV 490, MYCAV 491, 31848, 31849, 32328) are not represented.

Table 1. Background data of the examined *M. anserisalpingitidis* strains/field isolates (No. 1-97), and clinical specimens containing *M. anserisalpingitidis* DNA (No. 98-104).

No.	ID	Host	Sample source[a]	Country	Place[a]	Year	Other[a]	AB[b]	PCR[c]	MLST[d]
1	ATCC BAA-2147	goose	phallus lymph	Hungary	n.d.	1983		x	x	x
2	MYCAV 34	goose	phallus lymph	Hungary	Szentes	2011	Int	x		x
3	MYCAV 35	goose	phallus lymph	Hungary	Rém	2012		x		
4	MYCAV 36	goose	cloaca	Hungary	Hajdúböszörmény	2012		x		
5	MYCAV 38	goose	cloaca	Hungary	Kelebia	2012		x		x
6	MYCAV 39	goose	phallus lymph	Hungary	Rém	2012				x
7	MYCAV 40	goose	cloaca	Hungary	Rém	2012				x
8	MYCAV 44	goose	cloaca	Hungary	Nagykamarás	2012		x		x
9	MYCAV 47	duck	lung and air sac	Hungary	Tázlár	2012		x	x	x
10	MYCAV 49	goose	phallus lymph	Hungary	Tiszavasvári	2013		x		x
11	MYCAV 50	goose	phallus	Hungary	Cered	2013	Int	x		x
12	MYCAV 51	goose	phallus lymph	Hungary	Derekegyház	2013	Int	x		x
13	MYCAV 53	goose	phallus lymph	Hungary	Szentes	2013	Int	x		x
14	MYCAV 54	goose	follicle	Hungary	Hódmezővásárhely	2013	Int	x	x	x
15	MYCAV 55	goose	follicle	Hungary	Kiskunmajsa	2013		x		x
16	MYCAV 56	goose	phallus	Hungary	Sükösd	2013		x		x
17	MYCAV 59	goose	follicle	Hungary	Rém	2013		x		
18	MYCAV 61	goose	phallus lymph	Hungary	Tatárszentgyörgy	2013		x		x
19	MYCAV 62	goose	phallus and testis	Hungary	Rém	2013			x	
20	MYCAV 63	goose	trachea	Hungary	Sükösd	2013		x		x
21	MYCAV 65	goose	phallus lymph	Hungary	Rém	2014		x		x
22	MYCAV 66	goose	phallus lymph	Hungary	Tiszaföldvár	2014		x		x
23	MYCAV 67	goose	phallus lymph	Hungary	Szentes	2014	Int	x		x
24	MYCAV 68	goose	phallus lymph	Hungary	Érpatak	2014	Int	x		x
25	MYCAV 69	goose	phallus lymph	Hungary	Ludas	2014		x		x
26	MYCAV 70	goose	phallus lymph	Hungary	Cered	2014	Int	x		x
27	MYCAV 71	goose	phallus lymph	Hungary	Sükösd	2014		x		x
28	MYCAV 72	goose	phallus lymph	Hungary	Nagykamarás	2014		x		x
29	MYCAV 73	goose	cloaca	Hungary	Sükösd	2014				x
30	MYCAV 75	goose	phallus lymph	Hungary	Dömsöd	2014		x		x

Table 1 (continued). Background data of the examined *M. anserisalpingitidis* strains/field isolates (No. 1-97), and clinical specimens containing *M. anserisalpingitidis* DNA (No. 98-104).

No.	ID	Host	Sample source[a]	Country	Place[a]	Year	Other[a]	AB[b]	PCR[c]	MLST[d]
31	MYCAV 76	goose	phallus lymph	Hungary	Tiszabábolna	2014		x		x
32	MYCAV 77	goose	phallus lymph	Hungary	Sükösd	2014				x
33	MYCAV 78	goose	trachea	Hungary	Sükösd	2014				x
34	MYCAV 91	goose	phallus	Hungary	Hajdúsámson	2011		x		x
35	MYCAV 93	goose	phallus	Hungary	Bojt	2011		x		x
36	MYCAV 94	goose	cloaca	Hungary	Tiszabábolna	2012		x		x
37	MYCAV 160	goose	phallus lymph	Hungary	Érpatak	2015		x		
38	MYCAV 161	goose	phallus lymph	Hungary	Szilaspogony	2015		x		
39	MYCAV 162	goose	phallus lymph	Hungary	Encsencs	2015		x		
40	MYCAV 176	goose	phallus	Hungary	Cered	2015		x		
41	MYCAV 177	goose	phallus	Hungary	Cered	2015	Int, a	x		x
42	MYCAV 178	goose	follicle	Hungary	Cered	2015	Int, a	x		x
43	MYCAV 179	goose	trachea	Hungary	Apátfalva	2015		x	x	x
44	MYCAV 180	goose	phallus	Hungary	Kisbér	2015		x		x
45	MYCAV 202	goose	cloaca	Hungary	Kelebia	2015		x		x
46	MYCAV 203	goose	phallus lymph	Hungary	Kisbér	2015			x	
47	MYCAV 205	goose	phallus lymph	Hungary	Szentes	2015	Int, b			x
48	MYCAV 212	goose	phallus lymph	Hungary	Encsencs	2015	Int, c			x
49	MYCAV 218	goose	cloaca	Hungary	Encsencs	2015	Int, c			x
50	MYCAV 221	goose	cloaca	Hungary	Szentes	2015	Int, b		x	x
51	MYCAV 222	goose	cloaca	Hungary	Szentes	2015	Int, b			x
52	MYCAV 243	goose	cloaca	Hungary	Szentes	2016	Int, d			x
53	MYCAV 245	goose	phallus lymph	Hungary	Szentes	2016	Int, d		x	x
54	MYCAV 247	goose	phallus lymph	Hungary	Encsencs	2016	Int, e			x
55	MYCAV 248	goose	cloaca	Hungary	Encsencs	2016	Int, e			x
56	MYCAV 264	goose	semen	Hungary	Szentes	2016	Int			x
57	MYCAV 269	goose	follicle	Hungary	Dömsöd	2016			x	
58	MYCAV 270	goose	cloaca	Hungary	Szentes	2016	Int, f			x
59	MYCAV 271	goose	phallus lymph	Hungary	Szentes	2016	Int, f		x	x
60	MYCAV 275	goose	semen	Hungary	Gödöllő	2016			x	x

Table 1 (continued). Background data of the examined *M. anserisalpingitidis* strains/field isolates (No. 1-97), and clinical specimens containing *M. anserisalpingitidis* DNA (No. 98-104).

No.	ID	Host	Sample source[a]	Country	Place[a]	Year	Other[a]	AB[b]	PCR[c]	MLST[d]
61	MYCAV 313	goose	cloaca	Hungary	Boldogasszonyfa	2017				x
62	MYCAV 325	goose	cloaca	Hungary	Cered	2017	Int			x
63	MYCAV 327	goose	cloaca	Hungary	Szentes	2017	Int			x
64	MYCAV 332	goose	cloaca	Hungary	Cered	2017	Int			x
65	MYCAV 333	goose	cloaca	Hungary	Érpatak	2017	Int			x
66	MYCAV 340	goose	cloaca	Hungary	Hajdúsámson	2017				x
67	MYCAV 342	goose	trachea and serosa	Hungary	Hajdúböszörmény	2017	Int, g		x	x
68	MYCAV 343	goose	follicle	Hungary	Hajdúböszörmény	2017	Int, g		x	x
69	MYCAV 344	goose	cloaca	Hungary	n.d.	2012			x	
70	MYCAV 382	goose	cloaca	Hungary	Pusztaföldvár	2017				x
71	MYCAV 421	goose	cloaca	Hungary	Lapistó	2018			x	
72	MYCAV 423	goose	cloaca	Hungary	Szentes	2018	Int			x
73	MYCAV 424	goose	cloaca	Hungary	Encsencs	2018	Int			x
74	MYCAV 429	goose	cloaca	Hungary	Érpatak	2018	Int			x
75	MYCAV 430	goose	cloaca	Hungary	Cered	2018	Int			x
76	MYCAV 449	goose	trachea	Hungary	Derekegyház	2018	Int			x
77	MYCAV 452	goose	cloaca	Hungary	Sükösd	2018				x
78	MYCAV 493	goose	cloaca	Hungary	Hajdúsámson	2018	h			x
79	MYCAV 494	goose	phallus lymph	Hungary	Hajdúsámson	2018	h		x	x
80	MYCAV 498	goose	cloaca	Hungary	Encsencs	2018	Int			x
81	MYCAV 500	goose	cloaca	Hungary	Cered	2018	Int			x
82	MYCAV 502	goose	cloaca	Hungary	Érpatak	2018	Int			x
83	MYCAV 512	goose	cloaca	Hungary	Hajdúböszörmény	2018				x
84	MYCAV 665	goose	cloaca	Poland	Bydgoszcz	1985				x
85	MYCAV 666	goose	cloaca	Poland	Piotrków Trybunalski	1987				x
86	MYCAV 667	goose	cloaca	Poland	Kartuzy	1985				x
87	MYCAV 668	goose	cloaca	Poland	Starogard Gdański	1985				x
88	MYCAV 669	goose	cloaca	Poland	Rzeszów	1986				x
89	MYCAV 670	goose	cloaca	Poland	Olsztyn	1985				x
90	MYCAV 675	goose	cloaca	Poland	Zielona Góra	1986				x

Table 1 (continued). Background data of the examined *M. anserisalpingitidis* strains/field isolates (No. 1-97), and clinical specimens containing *M. anserisalpingitidis* DNA (No. 98-104).

No.	ID	Host	Sample source[a]	Country	Place[a]	Year	Other[a]	AB[b]	PCR[c]	MLST[d]
91	MYCAV 680	goose	cloaca	Poland	Piotrków Trybunalski	1985				x
92	MYCAV 783	goose	cloaca	China	Guangzhou	2019	i			x
93	MYCAV 785	goose	cloaca	China	Guangzhou	2019	i			x
94	MYCAV 903	goose	cloaca	Vietnam	Hanoi	2019				x
95	31848[e]	goose	oviduct	Hungary	n.d.	2003			x	
96	31849[e]	goose	ovum	Hungary	n.d.	2003			x	
97	32328[e]	goose	testis	Hungary	n.d.	2004			x	
98	PL 1	goose	cloaca	Poland	Olsztyn	1985				x
99	PL 2	goose	cloaca	Poland	Białystok	1985				x
100	PL 3	goose	cloaca	Poland	Białystok	1985				x
101	PL 5	goose	cloaca	Poland	Bydgoszcz	1985				x
102	PL 11	goose	cloaca	Poland	Piotrków Trybunalski	1986				x
103	PL 13	goose	cloaca	Poland	Piotrków Trybunalski	1986				x
104	UA 1	goose	phallus lymph	Ukraine	n.d.	2018				x
							Sum	39	19	89

[a]Abbreviations: Int – isolates are from the same livestock integration; a – MYCAV 177 and 178 are from the same flock; b – MYCAV 205 and 221 are from the same animal, and 222 from the same flock; c – MYCAV 212 and 218 are from the same animal; d – MYCAV 243 and 245 are from the same flock; e – MYCAV 247 and 248 are from the same animal; f – MYCAV 270 and 271 are from the same flock; g – MYCAV 342 and 343 are from the same flock; h – MYCAV 493 and 494 are from the same flock; i – MYCAV 783 and 785 are from the same flock; n.d. – no data

[b]AB: selected isolates for the antibiotic susceptibility study

[c]PCR: selected isolates for the species-specific PCR study

[d]MLST: selected isolates for the MLST study

[e]Examination was performed by Dmitriy V. Volokhov (Center for Biologics Evaluation and Research, U. S. Food and Drug Administration, USA)

Table 2. Background data of the examined *M. anatis* strains/field isolates.

No.	ID	Host	Sample source[a]	Country	Place[a]	Year[a]	PCR[b]	MLST[c]
1	NCTC 10156	duck	respiratory tract	United Kingdom	n.d.	1964	x	x
2	MYCAV 314	goose	cloaca	Hungary	Cered	2011	x	x
3	MYCAV 315	duck	lung and air sac	Hungary	Tázlár	2012	x	x
4	MYCAV 317	goose	peritoneum	Hungary	Kiskunfélegyháza	2013	x	x
5	MYCAV 318	duck	phallus lymph	Hungary	Szentes	2013	x	x
6	MYCAV 321	duck	phallus lymph	Hungary	Dévaványa	2014	x	x
7	MYCAV 324	duck	phallus lymph	Hungary	Szentes	2014	x	x
8	K6193A[d]	wild duck	n.d.	USA	New York State	n.d.	x	
9	K6193C[d]	wild duck	n.d.	USA	New York State	n.d.	x	

[a]Abbreviations: n.d. – no data
[b]PCR: selected isolates for the species-specific PCR study
[c]MLST: selected isolates for the MLST study
[d]Examination was performed by Dmitriy V. Volokhov (Center for Biologics Evaluation and Research, U. S. Food and Drug Administration, USA)

Table 3. Background data of the *M. anseris* strains/field isolates for the species-specific PCR evaluations.

No.	ID	Host[a]	Sample source[a]	Country	Place[a]	Year
1	ATCC 49234	goose	phallus lymph	Hungary	n.d.	1984
2	MYCAV 92	goose	phallus	Hungary	Hajdúsámson	2011
3	MYCAV 96	goose	oviduct	Hungary	Nyírábrány	2013
4	MYCAV 339	goose	cloaca	Hungary	Hajdúsámson	2017
5	MYCAV 346	goose	follicle	Hungary	Rém	2013
6	MYCAV 347	goose	phallus lymph	Hungary	Rém	2013
7	MYCAV 348	goose	phallus lymph	Hungary	Rém	2014
8	MYCAV 349	goose	cloaca	Hungary	Rém	2012
9	MYCAV 350	n.d.	n.d.	Hungary	Tiszabábolna	2014
10	MYCAV 451	goose	cloaca	Hungary	Cered	2018
11	MYCAV 454	goose	cloaca	Hungary	Szentes	2018
12	MYCAV 490	goose	cloaca	Hungary	n.d.	2018
13	MYCAV 491	goose	phallus lymph	Hungary	n.d.	2018
14	MYCAV 492	goose	cloaca	Hungary	Derekegyház	2018

[a]Abbreviations: n.d. – no data

Table 4. Background data of the *M. cloacale* strains/field isolates for the species-specific PCR evaluations.

No.	ID	Host[a]	Sample source[a]	Country	Place[a]	Year
1	NCTC 10199	turkey	cloaca	United Kingdom	n.d.	1975
2	MYCAV 335	goose	cloaca	Hungary	Érpatak	2017
3	MYCAV 336	goose	semen	Hungary	Boldogasszonyfa	2017
4	MYCAV 341	goose	cloaca	Hungary	Cered	2017
5	MYCAV 345	goose	cloaca	Hungary	Derekegyház	2017
6	MYCAV 351	goose	cloaca	Hungary	Hajdúböszörmény	2012
7	MYCAV 352	goose	phallus lymph	Hungary	Rém	2012
8	MYCAV 353	chicken	lung and trachea	Hungary	Rém	2012
9	MYCAV 354	goose	cloaca	Hungary	Rém	2012
10	MYCAV 355	goose	phallus	Hungary	Cered	2013
11	MYCAV 356	goose	cloaca	Hungary	Cered	2013
12	MYCAV 357	goose	phallus lymph	Hungary	Szentes	2014
13	MYCAV 358	goose	phallus lymph	Hungary	Dömsöd	2014
14	MYCAV 359	goose	phallus lymph	Hungary	Ludas	2014
15	MYCAV 360	goose	phallus lymph	Hungary	Cered	2014
16	MYCAV 361	n.d.	n.d.	Hungary	Tiszabábolna	2014
17	MYCAV 362	n.d.	follicle and oviduct	Hungary	Cered	2015
18	MYCAV 363	n.d.	phallus lymph	Hungary	Cered	2015
19	MYCAV 364	goose	cloaca	Hungary	Kelebia	2012

[a]Abbreviations: n.d. – no data

4.1.2. Culture method of waterfowl *Mycoplasma* strains/isolates

Tissue or swab samples were washed in 2 ml of Oxoid *Mycoplasma* broth medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) (pH 7.8) supplemented with 0.5% (w/v) sodium pyruvate, 0.5% (w/v) glucose, 0.15% L-arginine hydrochloride and 0.005% (w/v) phenol red, filtered through a 0.65 µm pore size syringe filter (Sartorius GmbH, Goettingen, Germany) and incubated at 37 °C. Swab samples were filtered as soon as possible; tissue samples were incubated at 37 °C for several hours or overnight before the filtering.

After the colour change, the cultures were inoculated onto solid Oxoid *Mycoplasma* medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 0.15% L-arginine hydrochloride and were incubated at 37 °C, 5% CO_2 until visible colonies appeared (1–4 days). Purification of mixed cultures was performed by one-time filter cloning, minimising the chance for the *in vitro* adaptation and mutations of the isolates.

The purity of the cultures (i.e. to exclude the contamination with other *Mycoplasma* spp.) was confirmed by the *Mycoplasma* genus-specific PCR system (Lauerman et al., 1995) followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA), sequence analysis and Basic Local Alignment Search Tool (BLAST) search. After the application of the newly developed species-specific PCR assays presented in the thesis, *Mycoplasma* species identification was based on the PCR results only, without sequencing.

The purified waterfowl *Mycoplasma* strains/isolates were cultured in the same media. Aliquots of cultures were stored at -70 °C until use.

4.2. Antibiotic susceptibility testing of *M. anserisalpingitidis*

4.2.1. Antimicrobial agents

The antimicrobial susceptibility testing was performed for isolated bacteria against seven classes of antibiotics including fluoroquinolones, aminoglycosides, lincosamides, tetracyclines, macrolides, pleuromutilins, and phenicols. Lincomycin and spectinomycin were applied in combination as well, in a ratio of 1:2 (Table 5).

Table 5. Antimicrobial agents for the susceptibility testing of *M. anserisalpingitidis* isolates.

Antibiotic class	Antibiotic agent	Manufacturer
Fluoroquinolones	Enrofloxacin	VETRANAL, Sigma-Aldrich, Germany
	Norfloxacin	
	Difloxacin	
Aminoglycosides	Spectinomycin	
Lincosamides	Lincomycin	
	Lincomycin-spectinomycin (1:2) combination	
Tetracyclines	Oxytetracycline	
	Doxycycline	
Macrolides	Tylosin	
	Tilmicosin	
	Tylvalosin	Aivlosin, ECO Animal Health Ltd., UK
Pleuromutilins	Tiamulin	VETRANAL, Sigma-Aldrich, Germany
	Valnemulin	
Phenicols	Florfenicol	

The antibiotics were diluted and stored according to the recommendations of Hannan (2000). Stock solutions of 1 mg/ml fluoroquinolones were prepared in 0.1 M NaOH; stock solution of 1 mg/ml florfenicol was prepared in 96% ethanol and sterile distilled water; the rest of the stock solutions of 1 mg/ml were prepared in sterile distilled water. Dilutions of the antibiotics were freshly prepared for each micro-test from the aliquots stored at −70 °C.

Two-fold dilutions were prepared in the range 0.039–10 µg/ml for fluoroquinolones, doxycycline and pleuromutilins, 0.25–64 µg/ml for spectinomycin, lincomycin, lincomycin-spectinomycin (1:2) combination, oxytetracycline and macrolides, and 0.125–32 µg/ml for florfenicol. The concentration ranges were selected based on antibiotic sensitivity studies of *M. synoviae* and *M. gallisepticum* (Wang et al., 2001; Gautier-Bouchardon et al., 2002; Behbahan et al., 2008; Gerchman et al., 2008; Landman et al., 2008; Forrester et al., 2011; Gharaibeh and Al-Rashdan, 2011; Lysnyansky et al., 2013).

4.2.2. Broth microdilution method

The broth microdilution examinations on 10^4–10^5 CCU/ml (CCU: colour changing unit) of the isolates were performed according to Hannan (2000). *Mycoplasma* broth medium (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used in the tests, and each 96-well microtitre plates contained growth controls (broth medium and *Mycoplasma* inoculum, without antibiotic), sterility controls (broth medium only, without antibiotic and *Mycoplasma* inoculum) and pH controls (broth medium adjusted to pH 6.8) (Figure 5).

The MIC values were determined from the lowest concentration of the antibiotics where no pH and colour change of the broth was detected, meaning that the growth of the

mycoplasmas was completely inhibited. The MIC values were recorded when colour change of the broth media of the growth control was visible. MIC_{50} and MIC_{90} values were defined as described by Hannan (2000).

At the time of the study, the research group did not possess the *M. anserisalpingitidis* type strain; therefore, a randomly chosen field isolate (MYCAV 65) was selected as the quality control of the MIC determination throughout the experiments. The antibiotic susceptibility profile of the ATCC BAA-2147 type strain was determined after the study, results were detailed in Table 16 only.

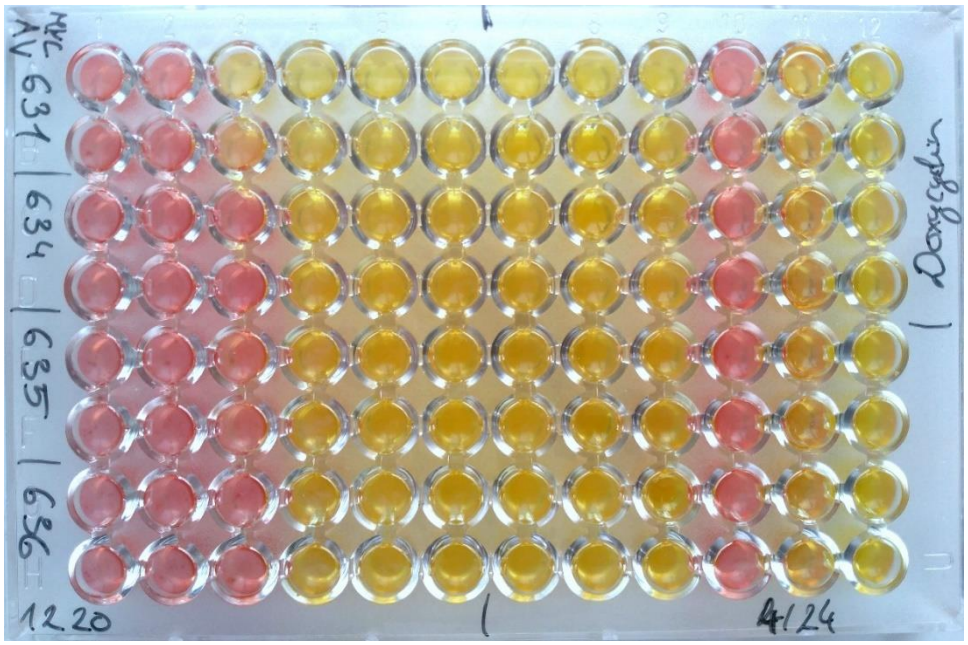

Figure 5. MIC determination of doxycycline with broth microdilution method against four *M. anserisalpingitidis* field isolates.
The columns are 1-9: descending concentrations of doxycycline (10-0.078 µg/ml); 10: sterility control; 11: endpoint control; 12: growth control. MIC values of doxycycline were 5 µg/ml against MYCAV 631 and 2.5 µg/ml against MYCAV 634, 635, and 363 isolates.

4.3. PCR assays designed for the studies

PCR assays were designed for the circularization of the generated contigs during whole genome sequencing, for the development of the species-specific PCR tests and for the *M. anserisalpingitidis* MLST assays. All the assays were carried out as described in Table 6.

Table 6. PCR reaction mixtures and thermocycling parameters for the circularization of the contigs, the species-specific PCR assays and the MLST study.

5X GoTaq Flexi Buffer	5 µl
$MgCl_2$ (25 mM) (Promega Inc., Madison, WI, USA)	2 µl
dNTP (10 mM) (Qiagen Inc., Hilden, Germany)	0.5 µl
Forward primer (10 pmol/µl)	2 µl
Reverse primer (10 pmol/µl)	2 µl
GoTaq G2 Flexi DNA polymerase (5 U/µl) (Promega Inc., Madison, WI, USA)	0.25 µl
nuclease-free water	to a final volume of 23 µl
DNA template	2 µl
Total volume	25 µl
Initial denaturation	95 °C for 5 min
Denaturation	95 °C for 50 sec
Annealing	61 °C for 1 min
Elongation	72 °C for 1 min
Cycle number	40
Final elongation	72 °C for 5 min

Detection of PCR products with the expected molecular weights was performed by electrophoresis in 1% TBE-agarose gel stained with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics Co., Ltd, New Taipei City, Taiwan), using GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA) weight marker, followed by UV visualization. Ultrapure water was used as a negative control, in parallel, to monitor for contamination.

4.4. *De novo* sequencing of waterfowl *Mycoplasma* strains/isolates

DNA was extracted from the ATCC BAA-2147, MYCAV 93, MYCAV 177, NCTC 10156, ATCC 4923, and NCTC 10199 strains/isolates. DNA library preparation kits (Illumina, Inc., San Diego, CA, USA), types of equipment (Illumina, Inc., San Diego, CA, USA), and generated reads are summarised in Table 7.

Table 7. Sequencing methods of waterfowl *Mycoplasma* strains/isolates.

Strain/Isolate	DNA library preparation kit	Illumina equipment	Generated reads[a]
M. anserisalpingitidis ATCC BAA-2147	TruSeq LT	MiSeq	2 × 250 bp MP
M. anserisalpingitidis MYCAV 93	Nextera mate pair	NextSeq 500	2 × 150 bp MP
	Nextera XT	NextSeq 500	2 × 75 bp PE
M. anserisalpingitidis MYCAV 177	TruSeq LT	MiSeq	2 × 250 bp MP
	Nextera XT	NextSeq 500	2 × 75 bp PE
M. anatis NCTC 10156	Nextera mate pair	NextSeq 500	2 × 150 bp MP
	Nextera mate pair	NextSeq 500	2 × 75 bp MP
M. anseris ATCC 49234	Nextera mate pair	NextSeq 500	2 × 150 bp MP
	Nextera mate pair	NextSeq 500	2 × 75 bp MP
M. cloacale NCTC 10199	Nextera mate pair	NextSeq 500	2 × 150 bp MP
	Nextera mate pair	NextSeq 500	2 × 75 bp MP

[a]Abbreviations: MP – mate-pair; PE – paired-end

The NxTrim software (O'Connell et al., 2015), with default settings, was used to trim the junction adapters from all of the raw mate-pair (MP) reads, generating shorter paired-end (PE) reads as well. First, contigs were generated for each strain/isolate from all of the PE output data using the SPAdes Genome Assembler 3.11 (Bankevich et al., 2012) with the "assembly only" option. Then, the PE contigs and the trimmed MP output data were assembled with the same option, generating the draft genomes. Trimmed reads (MP and PE) were control mapped to the draft *de novo* genome and curated with Geneious 9.1.8 software (Kearse et al., 2012).

The circularization of the contigs was performed by specific primer pairs designed for the contigs' ends, and the PCR products were sequenced on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed with the two primers (forward and reverse); however, the two sequences did not always overlap. In that case, 5' and 3' ends of a contig were extended, and primer design and sequencing were performed again. The overlapped sequences revealed the absent fragments, and the fragments were added to the contigs in the Geneious software to create the circularized complete genomes (Table 8). Primer design was performed using the NetPrimer software (http://www.premierbiosoft.com/netprimer/). The circularization assays were carried out as described in Table 6.

Table 8. Primer sequences for the circularization of the waterfowl *Mycoplasma* contigs.

Strain/Isolate	Test	Primer sequence (5'→3')	Size of amplicon (bp)	Result	
				extended contig[a]	circularized genome[b]
M. anserisalpingitidis ATCC BAA-2147	1	CCTATTACCAAGTCTCATAAAATTCCG TTGGTAGATTTTTTGAGAAAAGAAGC	1534	x	
	2	GGCTGAACAAGAGTTAAATATAGAAATG TTACAAAACTCAATTTTTGGTGCC	497		x
M. anserisalpingitidis MYCAV 93	1	GAATTTCCTAATGGTTTAATCTCTTGTA ACTTATACTCACTTGGTGTAATGGATGA	2641	x	
	2	TTAACTCTTGTTCAGCCATTTCTCTA CAGAAGCAGTTAAAGATGGCGTT	1350		x
M. anserisalpingitidis MYCAV 177		AGTGATATCTAAATCAAGAATTTAAGTATATTAAA ATCATGGCGTTGAAGCTACTCTT	1116		x
M. anatis NCTC 10156		ATTTTTTAGAATTTGTATATTCCAATGTTAG TAATATATGTGAAATTATACAAGATTATCAATGA	602		x
M. anseris ATCC 49234		AAAAACACCTGTTAGAGATGTATTAGCA CGTTTCTTGATTGCTCCACCA	400		x
M. cloacale NCTC 10199	1	TATGAATATAGTGGAGGTCATGTTTTATG AAAAATGGAAAATAAAGTCAAAACTTTC	1752	x	
	2	CACAAGATCTATTATTTGAAAATAAACATAAA AATTGATGAGAAGCTGACAAGAAAC	661		x

[a]Sequencing was performed with the two primers; however, the two sequences did not overlap but the contig's 5' and 3' ends could be extended.
[b]Sequencing was performed with the two primers. After that, the sequences revealed the fragment between the contig's 5' and 3' ends.

The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016) online service was used to annotate all six genomes. Moreover, the rRNA, tRNA and tmRNA genes were verified by RNAmmer (Lagesen et al., 2007) and ARAGORN (Laslett and Canback, 2004) softwares in the genomes. The genes were classified into functional role categories as defined by the SEED classification of the Rapid Annotations using Subsystems Technology (RAST) software after in-software annotation (Aziz et al., 2008).

Sequences from shotgun projects of *M. anatis*, *M. anseris*, and *M. cloacale* type strains (accession numbers AFVJ00000000, JNJX00000000 and JNJL00000000, respectively) were aligned to the generated complete genomes of the corresponding type strains with Geneious 9.1.8 software (Kearse et al., 2012).

Whole genome comparisons were performed using Mauve version 2.3.1 software (Darling et al., 2004) integrated into the Geneious software as follows: (i) *M. anserisalpingitidis* ATCC BAA-2147 type strain with MYCAV 93 and MYCAV 177, (ii) *M. anserisalpingitidis* ATCC BAA-2147 with *M. anatis* NCTC 101156, and (iii) *M. anseris* ATCC 49234 with *M. cloacale* NCTC 10199.

4.5. Development of species-specific PCR assays

4.5.1. Selection of species-specific regions

In order to design species-specific primers for the detection of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale*, several housekeeping genes were selected randomly from the *Mycoplasma* minimal genome set (Razin et al., 1998; Liu et al., 2012), which are presented in the genomes of the examined species. The sequences of the selected genes were obtained from the complete genomes of *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* type strains. The selected genes are listed in Table 9. At the time of the study, the genomes' annotation was not performed with the NCBI PGAP; therefore, the annotation was performed using the RAST software (Aziz et al., 2008). Based on the genome analysis, all these genes are presented as a single copy in the genomes of these four mycoplasmas.

Table 9. List of the genes analysed for design of species-specific primers.

No.	Gene	Gene function	*M. anserisalpingitidis* ATCC BAA-2147[a]	*M. anatis* NCTC 10156[a]	*M. anseris* ATCC 49234[a]	*M. cloacale* NCTC 10199[a]
1	*ackA*	acetate/propionate family kinase	x	x	x	x
2	*arcA*	arginine deiminase			x	x
3	*cdd*	cytidine deaminase	x	x	x	x
4	*comEB*	deoxycytidylate deaminase		x	x	
5	*dnaX*	DNA polymerase III subunit gamma/tau	x	x	x	x
6	*gtaB*	UTP-glucose-1-phosphate uridylyltransferase	x	x		
7	*mraZ*	transcriptional regulator	x	x	x	x
8	*mtn*	5'-methylthioadenosine nucleosidase			x	x
9	*pcrA*	ATP-dependent DNA helicase	x	x	x	x
10	*pfkB*	1-phosphofructokinase	x	x		
11	*pgiB*	glucose-6-phosphate isomerase	x	x	x	x
12	*pgmB*	beta-phosphoglucomutase	x	x		
13	*ptsP*	phosphoenolpyruvate-protein phosphotransferase	x	x		
14	*rpe*	ribulose-phosphate 3-epimerase	x	x	x	x
15	*rpoB*	DNA-directed RNA polymerase subunit beta	x	x	x	x
16	*thyA*	thymidylate synthase		x	x	

[a]Crossmark means that the selected gene is presented in the concerned genome.

4.5.2. Primer design

The sequences of the selected genes were aligned and analysed using the Geneious software (Kearse et al., 2012). The genes were manually analysed and the primer pairs were designed according to the following three main acceptance criteria: (i) the gene of interest should have at least two regions (20–30 bp) containing as many species-specific nucleotide substitutions as possible, and these short sequence regions should be suitable for the design of species-specific primers, i.e., to have similar melting temperatures, and do not form a hairpin, or self- and cross-dimers, (ii) the distance between the primers' regions should be approx. 500–1000 bp, and (iii) the selected species-specific primers should be able to amplify the target gene from all tested field isolates of the same species but not from others. The primer design was performed using the NetPrimer software (http://www.premierbiosoft.com/netprimer). The specificity of the primers was analysed *in silico* using BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.5.3. Development of the assays

To evaluate the PCR assays, DNA samples of waterfowl *Mycoplasma* field isolates (Tables 1-4) and clinical specimens were used (Table 19). The clinical specimens were primarily screened for the presence of *Mycoplasma* DNA using the genus-specific PCR (Lauerman et al., 1995). The species-specific PCR assays were carried out as described in Table 6. The final primer set was selected based on specificity and sensitivity criteria detailed in the next section. The sequences of the designed primers are provided in Tables 10 and 11.

Table 10. Designed primers for the waterfowl *Mycoplasma* species-specific PCR assays.

***Mycoplasma* species**	**Target gene**	**Forward primer (5' - 3')[a]**	**Reverse primer (5' - 3')[a]**
M. anserisalpingitidis	*rpoB*	**CCGTGATACTGCTCAATTCGAA**	**TAGAAGTATAAACATCATCCTTAACAAGCT**
		AAATTAACCCTCTAGCTGAAGTGTCA	GACCTACTAAAACATCACCAGGAACT
	pfkB	CAACCTAAGAAGTTTATATGACATGCTTT	TTGACTTGCTTTTTCTACAATTTCTTC
	pgmB	GTACCAGTTCCTTCAGACGTTATTTT	GCATTTTCTAATTCATTAAAATCTAAATCTG
			GAATGAATTGATTTAAGTCCTTCAACA
M. anatis	*dnaX*	**CAGAGATCAGTCTGTTTTAGAATTACTTT**	**TTTCTCAGATGCTTGTGAAATACAACTT**
	pgmB	TTAGCATGAAAAGAAATAGTAGCTGAATTA	GAAACAATAGAGTTTAATCCTTCCACC
	ptsP	TTTACAACAGAAAGAATTATTGATATGATC	TTTGAAGAGATAAGTTAGAAAGCATCT
	thyA	CTGATCGTACAGGAGTAGGTACAATTAGTA	GTTAGGATTTAGTTTTACAATAGGTAATTCTAG
M. anseris	*dnaX*	TCAAATTCAAAAATTGTTCCTTGC	ATGTGTTCTAATTGAAGCCATTTTAAT
	pcrA	**CTAAAAACTCCTAAAGACTTAGAAGAATC**	**ATCCTCACCTTCATCATTTTCTGTATA**
	mtn	AAGAATCTGAATTTATTTTAAGTCATGTTG	TGCTGATAATCGTTAGGATTAGAAAAA
M. cloacale	*dnaX*	**TTCATCCGATAAGTTAAAACCTTGTT**	**AAAACTGCTTTTGTATTTTTAGAATATAGT**
			TATTTATTTCTTCAACATAAATATATGCAA
	mtn	AAGAATACCAATTAAACAATATCTATAACGAT	TGTTATGACTTATTAAAGAATCTGAAACAA

[a]Final primer sequences are bolded and detailed in Table 11.

Table 11. List of the primer sequences and sizes of amplicons used for species-specific PCR assays.

***Mycoplasma* species**	**Target gene**	**Primer sequence (5'-3')**	**Size of amplicon (bp)**
M. anserisalpingitidis	*rpoB*	CCGTGATACTGCTCAATTCGAA TAGAAGTATAAACATCATCCTTAACAAGCT	857
M. anatis	*dnaX*	CAGAGATCAGTCTGTTTTAGAATTACTTT TTTCTCAGATGCTTGTGAAATACAACTT	895
M. anseris	*pcrA*	CTAAAAACTCCTAAAGACTTAGAAGAATC ATCCTCACCTTCATCATTTTCTGTATA	504
M. cloacale	*dnaX*	TTCATCCGATAAGTTAAAACCTTGTT AAAACTGCTTTTGTATTTTTAGAATATAGT	591

4.5.4. Assessment of the specificity and sensitivity of the developed assays

The specificity of the PCR assays was analysed using the following avian *Mycoplasma* and *Acholeplasma* type strains: *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234), *M. anserisalpingitidis* (ATCC BAA-2147), *M. cloacale* (NCTC 10199), *M. columbinasale* (ATCC 33549), *M. columborale* (ATCC 29258), *M. gallinaceum* (ATCC 33550), *M. gallinarum* (ATCC 19708), *M. gallisepticum* (ATCC 19610), *M. gallopavonis* (ATCC 33551), *M. iners* (ATCC 19705), *M. iowae* (ATCC 33552), *M. meleagridis* (NCTC 10153), *M. pullorum* (ATCC 33553), *M. synoviae* (NCTC 10124) and *Acholeplasma laidlawii* (NCTC 10116).

In order to test the sensitivity of the PCR assays, all designed primer pairs were tested with the corresponding DNA of the *M. anserisalpingitidis* (ATCC BAA-2147), *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234) and *M. cloacale* (NCTC 10199) strains serially diluted 10-fold (10^6–10^0) in nuclease-free water. The template copy numbers (corresponding to genomic equivalents, GE) were calculated based on the used DNA concentrations measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), the genome size of the strains (Table 17) and an online dsDNA copy number calculator (https://cels.uri. edu/gsc/cndna.html).

During the selection of the final primer set, primers which showed cross-amplification between the tested *Mycoplasma*/*Acholeplasma* strains/isolates were excluded. The sensitivity of the PCR assays using different primer pairs for the detection of a certain waterfowl *Mycoplasma* species was compared visually with gel electrophoresis. Primer pairs showing the highest sensitivity and specificity were chosen for the final study.

In order to evaluate the performance of the designed assays in mixed infections, the DNA of the waterfowl *Mycoplasma* type strains (containing 10^6 GE) were mixed in a ratio of

1:1:1:1 and tested. Also, DNA mixes were created with one DNA sample containing 10^3 GE and the other three are represented with 10^6 GE in a ratio of 1:1:1:1, and submitted to the corresponding waterfowl *Mycoplasma*-specific assay (i.e. DNA mix of 10^3 GE *M. anserisalpingitidis*, 10^6 GE *M. anatis*, 10^6 GE *M. anseris* and 10^6 GE *M. cloacale* in the *M. anserisalpingitidis*-specific PCR assay).

4.5.5. Confirmation of the assays' specificity by PCR amplicon sequencing

In order to demonstrate that the obtained PCR products were species-specifically amplified, direct DNA sequencing was performed on all amplicons from the type strains and amplicons generated from some selected field isolates and clinical specimens with an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). In the case of the *M. anserisalpingitidis*-specific PCR, the product of the MYCAV 47 isolate originating from a duck, and a randomly selected amplicon (clinical specimen No. 21, Table 19) were sequenced to confirm their species identity. All PCR products amplified from goose specimens using the *M. anatis*-specific PCR assay (i.e., MYCAV 314 and 317 isolates and clinical specimens No. 2, 3, 5, 7, Table 19) were also sequenced to confirm their species identity because the presence of *M. anatis* in geese is not commonly reported. Randomly selected amplicons were also sequenced from the *M. anseris*- and *M. cloacale*-specific PCRs to verify their species identity (clinical specimens No. 1 and 6, respectively, Table 19).

The *Mycoplasma* genus-specific PCR products of clinical specimens No. 1, 13, 25 and 28, which according to the results of the developed species-specific PCR assays represented mixed mycoplasma DNA samples due to natural mycoplasma co-infections in geese, were also sequenced to analyse their DNA sequence chromatograms for the presence of mixed sequences (Table 19).

The sequences obtained from the type strains, field isolates and clinical specimens were deposited in GenBank under accession numbers MK532897–MK532910.

4.6. MLST study of *M. anserisalpingitidis*

4.6.1. Whole-genome sequencing

Next-generation sequencing was performed on the DNA samples of 82 *M. anserisalpingitidis* and six *M. anatis* isolates (Table 1 and 2) with NextSeq 500 Illumina equipment (Illumina, Inc., San Diego, CA, USA), using NextSeq 500/550 High Output Kit v2.5 reagent kit (Illumina, Inc., San Diego, CA, USA) (Kovács et al., 2020). The obtained sequences were mapped to the appropriate reference genome of *M. anserisalpingitidis* ATCC BAA-2147 and *M. anatis* NCTC 10156 using the Geneious Prime 2019.2.1 software (Kearse et al., 2012).

4.6.2. Selection of housekeeping genes for MLST

Twenty-eight housekeeping gene fragments were selected from previously published *Mycoplasma* MLST studies (Mayor et al., 2008; McAuliffe et al., 2011; Manso-Silván et al., 2012; Tocqueville et al., 2014; Dijkman et al., 2016; Ghanem and El-Gazzar, 2016; El-Gazzar et al., 2017; Bekő et al., 2019), and further 12 randomly selected genes, represented in the genome of the *M. anserisalpingitidis* ATCC BAA-2147 type strain were added to the analyses (Table 12). The sequences of the selected genes were obtained from the genomes of the ATCC BAA-2147 type strain, and the 81 *M. anserisalpingitidis* field isolates. The selected MLST loci were obtained from the *M. anatis* NCTC 10156 type strain and the whole-genome sequences of six field isolates, and were included in the analyses, too. Criteria for the selection of the housekeeping genes were in accordance with previous publications (Mayor et al., 2008; McAuliffe et al., 2011; Manso-Silván et al., 2012; Tocqueville et al., 2014; Dijkman et al., 2016; Ghanem and El-Gazzar, 2016; El-Gazzar et al., 2017; Bekő et al., 2019) and are summarised as follows: (i) the selected genes are present in all *M. anserisalpingitidis* genomes, (ii) the selected genes possess highly diverse internal fragments surrounded by conserved regions suitable for primer design, (iii) the selected fragments show high Simpson's index of diversity, (iv) the amplicon sizes of the selected gene fragments are between 300-600 bp, suitable for Sanger sequencing, (v) the genes are evenly distributed in the genome to limit the effect of mutation events, and (vi) preferably PCR primers are species-specific. Simpson's index of diversity for the selection of MLST loci was calculated based on the sequence types of 82 isolates via an online tool available from the Comparing Partitions website (http://www.comparingpartitions.info/index.php?link=Tool). The selected housekeeping genes were aligned using the Geneious Prime 2019.2.1 software (Kearse et al., 2012) and the primer design was performed using the NetPrimer software (http://www.premierbiosoft.com/netprimer). Primer pairs previously described for the genetic identification of *M. anserisalpingitidis* (Sprygin et al., 2012) (Table 13) were also investigated by *in silico* analysis using the Geneious Prime 2019.2.1 software (Kearse et al., 2012).

Table 12. Background information of the genes examined during the development of the MLST scheme of *M. anserisalpingitidis.*

No.	Gene	Gene function	Selection based on[a]
1	*atpG*	F0F1 ATP synthase subunit gamma	reported in g, h
2	*fusA*	elongation factor G	reported in c, h
3	*pgiB*	glucose-6-phosphate isomerase	reported in a
4	*plsY*	glycerol-3-phosphate acyltransferase	random selection
5	*uvrA*	excinuclease ABC subunit	reported in e, h
6	*dnaA*	chromosomal replication initiator	reported in b, d, h
7	*efp*	elongation factor P	reported in a, g
8	*gmk*	guanylate kinase	reported in a, d, g
9	*leuS*	leucine-tRNA ligase	reported in f
10	*pyrH*	UMP kinase	random selection
11	*rpoB*	DNA-directed RNA polymerase subunit beta	reported in a, c, d, h
12	*ruvB*	Holliday junction branch migration DNA helicase	reported in e, h
13	*tpiA*	triose-phosphate isomerase	reported in a
14	*metG*	methionine-tRNA ligase	reported in a
15	*ugpA*	ABC transporter, permease protein	reported in e
16	*ackA*	acetate/propionate family kinase	random selection
17	*adk*	nucleoside monophosphate kinase	reported in a, d, g
18	*alaS*	alanine-tRNA ligase	random selection
19	*algA*	alpha-amylase	random selection
20	*argS*	arginine-tRNA ligase	random selection
21	*aspS*	aspartate-tRNA ligase	random selection
22	*calB*	aldehyde dehydrogenase family protein	random selection
23	*dnk*	deoxynucleoside kinase	random selection
24	*gltX*	glutamate-tRNA ligase	reported in b, d
25	*gyrB*	DNA gyrase subunit B	reported in a, b, c, d
26	*lepA*	elongation factor 4	reported in c, e
27	*pfkA*	6-phosphofructokinase	random selection
28	*ppa*	inorganic diphosphatase	reported in g
29	*recA*	recombinase	reported in a, g
30	*rnr*	ribonuclease R	random selection
31	*rpoC*	DNA-directed RNA polymerase subunit beta'	reported in f
32	*tufA*	elongation factor Tu	reported in b
33	*valS*	valine-tRNA ligase	reported in f
34	*pfkB*	1-phosphofructokinase	random selection
35	*dppC*		reported in f
36	*kdpA*		reported in f
37	*metS*	methionyl-tRNA synthetase	reported in b
38	*nagC*		reported in g
39	*nanA*		reported in e
40	*ulaA*		reported in f
41	*dnaE*	DNA-polymerase III subunit alpha	reported in i
42	*fusA*	elongation factor G	reported in i
43	*pyk*	pyruvate kinase	reported in i
44	*rpoB*	DNA-directed RNA polymerase subunit beta	reported in i

[a]Abbreviations: a – Mayor et al., 2008; b – McAuliffe et al., 2011; c – Manso-Silván et al., 2012; d – Tocqueville et al., 2014; e – Dijkman et al., 2016; f – Ghanem and El-Gazzar, 2016; g - El-Gazzar et al., 2017; h – Bekő et al., 2019; i – Sprygin et al., 2012

4.6.3. Phylogenetic analysis

For each MLST locus, all sequences were compared, and allele numbers were assigned to each unique allele variant. The samples were grouped into STs according to the allelic numbers of the five loci. The discriminatory power of the method was calculated using Simpson's index of diversity with 95% confidence intervals (Hunter and Gaston, 1988).

Internal fragments (i.e. primer sequences were not included) of the selected housekeeping genes were aligned and concatenated using Geneious Prime 2019.2.1 (Kearse et al., 2012). Phylogenetic analysis of the concatenated sequences was performed using MEGA X 10.0.5 (Kumar et al., 2018). The evolutionary history with the *M. anatis* outgroup was inferred using the Maximum Likelihood method, based on Tamura-Nei model (Saitou and Nei, 1987) with 1000 bootstrap.

4.6.4. PCR conditions, specificity and sensitivity tests

All PCR reactions were carried out as described in Table 6. Table 13 and Table 14 summarise primers used in this study.

Table 13. List of the primer sequences used in the MLST study.

No.	Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
1	*atpG*	Listed in Table 14	
2	*fusA*		
3	*pgiB*		
4	*plsY*		
5	*uvrA*		
6	*dnaA*	GGAATAGTTTTTTCTGTCGATTCTG	TGAACACCCTTAAGCAAATGAGTTT
7	*efp*	TGGTATAACATTCCAAGATGAAGGTG	CTTCTTCCGACGTATTTACCAGTTT
8	*gmk*	GAAAACTCCTTTAGTTATATTTTCTGGA	GTGATTGTCTTCAGAACCTCTTTTTAAT
9	*leuS*	ACGTTAGAAACTATTCCCTTGGTGAT	CATTTAGAAGTTCTTCTGCATAATCAG
10	*pyrH*	AGAAGTGGTTTTGAACAACATGGAT	TTTCGTCATAAGTAATTTTATCAAAACG
11	*rpoB*	AAATTGAGTGTTGTTGACCGTATTGT	AGGATCATCATCAGTACCAATTCCAT
12	*ruvB*	CCAAGTAGTTTTGATGAATTTATTGGTC	GCAATTCTTGGAGTGAAGTTAGAAAAT
13	*tpiA*	GTTGATCTTGGTGCAAAATATGTAATT	CAACTAAGAATCCATCAATACTTGGTT
14	*metG*	GCATGAACTCTTGCTAATTACAAAAA	ACCATCATCTTTAAGAACTGCTTGAG
15-40		Primer design was not performed	
41	*dnaE*	CAAACATTAGGAGCAAAGAATTCG[a]	TGGTTTCGCTAATTTCTGGAT[a]
42	*fusA*	ACATTATTGATACACCAGGGCACG[a]	GGTTCAAGAGCTTGTGAAATAACTGG[a]
43	*pyk*	ACTTCAGTCATGGAGATCACTC[a]	TCAAGTGGGAATTTACCGTTTGCAG[a]
44	*rpoB*	TAACCAAATTCCAATTGTAAAACTTGGTG[a]	CAAGAACATCACCAAGTTCTCTAC[a]

[a]Reported in Sprygin et al., 2012

Table 14. Amplicon sizes, analysed sequence fragment lengths and primer sequences of the target regions for the five loci studied in *M. anserisalpingitidis.*

Target gene	Primer sequence (5'-3')	Size of amplicon (bp)	Length of internal fragment (bp)
atpG	TCCTGTGGATAAATCAAACGAAAG GAAAGTTTTGAAGCTGCACCTAAG	498	450
fusA	AGCTTTCACATATAATGGGGAAGC CTGATTTTTCTGAAAGTAATGTATCTCC	629	577
pgiB	CACAAAAAAGACCAGACATGGAATT ATAAACCTACTGCTGTCATAACTGAGA	336	284
plsY	AGTCATAAAAGAAAATCACAAGACATTC TTGCGAGTGAAACATATCTTGTGATA	379	325
uvrA	TTGCTTTCAAGATTTCTAATGGTTTAC CGCTTGTTGAAGTTAAAACATATTCA	426	373

In the case of the clinical specimens, *M. anserisalpingitidis* DNA was identified in the samples with the help of the species-specific PCR assay. The presence of *M. anatis* DNA was ruled out with the species-specific PCR assay, too. Partial sequences of the MLST housekeeping genes from these samples were obtained with primers listed in Table 14, by direct sequencing of the produced amplicons on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The specificity of the PCR assays was assessed by testing DNA extracts from avian *Mycoplasma* type strains: *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234), *M. anserisalpingitidis* (ATCC BAA-2147), *M. cloacale* (NCTC 10199), *M. gallinarum* (ATCC 19708), *M. gallisepticum* (ATCC 19610), *M. iners* (ATCC 19705), and *M. synoviae* (NCTC 10124). The PCR sensitivity was evaluated in the same way as described in section 4.5.4.

4.6.5. Nucleotide sequence accession numbers

Partial sequences of the five housekeeping genes of 79 novel *M. anserisalpingitidis* field isolates (complete genomes were uploaded for ATCC BAA-2147, MYCAV 93 and MYCAV 177, see in Table 17) and seven clinical specimens were deposited in GenBank under accession numbers MN722655 – MN722737, and MT501658 – MT501660 for *atpG*, MN722904 – MN722986, and MT501664 – MT501666 for *fusA*, MN722738 – MN722820, and MT501661 – MT501663 for *pgiB*, MN722987 – MN723069, and MT501667 – MT501669 for *plsY*, MN723070 – MN723152, and MT501670 – MT501672 for *uvrA*. Online database for this MLST scheme is available at https://pubmlst.org/manserisalpingitidis/. The sequences of the six *M. anatis* field isolates were submitted to GenBank under the accession numbers MT508595 – MT508618, and MT508625 – MT508630.

5. Results

5.1. Antibiotic susceptibility profile of *M. anserisalpingitidis*

The quality control isolate (MYCAV 65) showed consistent results throughout the study. Field isolates with elevated MIC values were found in the cases of all tested antibiotics. The MIC ranges, MIC_{50} and MIC_{90} values are summarised in Table 15. For detailed MIC values see Table 16.

Table 15. Summary of MIC range, MIC_{50} and MIC_{90} values of the examined *M. anserisalpingitidis* isolates.

Antibiotic class	Antibiotic agent	Range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Fluoroquinolones	Enrofloxacin	1.25 to >10	5	>10
	Norfloxacin	10 to >10	>10	>10
	Difloxacin	1.25 to >10	10	>10
Aminoglycoside	Spectinomycin	4 to >64	8	32
Lincosamide	Lincomycin	2 to >64	4	8
	Lincomycin-spectinomycin (1:2) combination	2 to 32	4	4
Tetracyclines	Oxytetracycline	2 to >64	64	>64
	Doxycycline	0.078 to >10	5	>10
Macrolides	Tylosin	≤0.25 to >64	8	>64
	Tilmicosin	≤0.25 to >64	>64	>64
	Tylvalosin	≤0.25 to 16	0.5	4
Pleuromutilins	Tiamulin	0.156 to 5	0.625	1.25
	Valnemulin	≤0.039 to 0.312	≤0.039	0.078
Phenicol	Florfenicol	2 to 32	8	8

Table 16. MIC values of the analysed *M. anserisalpingitidis* strains/field isolates.

	MIC (µg/ml)													
ID	**Enrofl[a]**	**Norfl[a]**	**Difl[a]**	**Spect[a]**	**Linko[a]**	**LiSp[a]**	**Oxy[a]**	**Doxy[a]**	**Tylo[a]**	**Tilmi[a]**	**Tilva[a]**	**Tia[a]**	**Valne[a]**	**Florf[a]**
ATCC BAA-2147	1.25	>10	2.5	32	2	4	>64	>10	0.5	≤0.25	≤0.25	1.25	≤0.039	8
MYCAV 34	5	>10	10	8	4	2	64	2.5	1	4	≤0.25	0.625	≤0.039	4
MYCAV 35	5	>10	10	>64	4	4	64	10	1	4	≤0.25	1.25	≤0.039	8
MYCAV 36	5	>10	>10	64	4	4	64	>10	1	4	≤0.25	1.25	≤0.039	8
MYCAV 38	2.5	>10	10	8	2	4	4	0.156	≤0.25	≤0.25	≤0.25	0.625	≤0.039	4
MYCAV 44	5	>10	10	8	4	4	8	0.312	8	>64	0.5	1.25	≤0.039	8
MYCAV 47	>10	>10	>10	16	>64	16	>64	5	16	>64	1	2.5	0.312	8
MYCAV 49	5	>10	10	16	4	4	64	5	8	>64	0.5	0.625	≤0.039	8
MYCAV 50	>10	>10	>10	16	4	4	>64	5	2	2	≤0.25	0.625	≤0.039	8
MYCAV 51	5	>10	10	32	4	4	>64	10	8	>64	0.5	0.625	≤0.039	8
MYCAV 53	5	>10	10	16	4	4	>64	10	8	>64	0.5	0.625	≤0.039	8
MYCAV 54	5	>10	10	8	4	4	>64	5	8	>64	0.5	0.625	≤0.039	8
MYCAV 55	10	>10	10	8	4	4	8	0.312	≤0.25	≤0.25	≤0.25	0.625	≤0.039	4
MYCAV 56	1.25	>10	1.25	8	4	4	4	0.312	8	>64	0.5	0.625	≤0.039	4
MYCAV 59	5	>10	10	8	4	4	32	2.5	0.5	≤0.25	≤0.25	1.25	0.078	2
MYCAV 61	5	>10	10	16	2	4	2	0.078	≤0.25	≤0.25	≤0.25	0.312	≤0.039	4
MYCAV 63	1.25	10	1.25	8	2	2	4	0.312	4	64	≤0.25	0.156	≤0.039	4
MYCAV 65	5	>10	10	16	4	4	32	5	0.5	0.5	≤0.25	1.25	0.078	8
MYCAV 66	5	>10	10	16	4	4	>64	>10	≤0.25	≤0.25	≤0.25	0.625	≤0.039	4
MYCAV 67	5	>10	10	8	>64	16	>64	5	>64	>64	16	2.5	0.078	4
MYCAV 68	5	>10	10	8	>64	32	>64	10	>64	>64	16	5	≤0.039	8
MYCAV 69	5	>10	10	4	4	4	>64	5	8	>64	1	0.625	≤0.039	4
MYCAV 70	>10	>10	>10	16	4	4	>64	>10	16	>64	1	0.625	≤0.039	8
MYCAV 71	1.25	>10	1.25	8	2	4	8	0.625	8	>64	0.5	0.625	≤0.039	4
MYCAV 72	5	>10	10	8	4	4	4	0.312	8	>64	0.5	0.625	≤0.039	4
MYCAV 75	5	>10	10	16	4	4	>64	10	≤0.25	≤0.25	≤0.25	0.625	≤0.039	8
MYCAV 76	5	>10	10	32	8	4	64	5	8	>64	0.5	1.25	≤0.039	8
MYCAV 91	10	>10	>10	8	8	4	64	2.5	≤0.25	≤0.25	≤0.25	0.625	≤0.039	8
MYCAV 93	2.5	>10	1.25	8	2	4	8	0.312	≤0.25	≤0.25	≤0.25	0.312	≤0.039	8
MYCAV 94	2.5	>10	5	16	4	4	>64	>10	≤0.25	≤0.25	≤0.25	0.625	≤0.039	8
MYCAV 160	>10	>10	>10	16	4	4	>64	10	>64	>64	2	0.625	≤0.039	8
MYCAV 161	>10	>10	>10	8	4	4	>64	>10	16	>64	0.5	0.625	≤0.039	8
MYCAV 162	2.5	>10	10	8	4	4	>64	5	16	>64	0.5	0.625	≤0.039	4

Table 16 (continued). MIC values of the analysed *M. anserisalpingitidis* strains/field isolates.

	MIC (µg/ml)													
ID	**Enrofl[a]**	**Norfl[a]**	**Difl[a]**	**Spect[a]**	**Linko[a]**	**LiSp[a]**	**Oxy[a]**	**Doxy[a]**	**Tylo[a]**	**Tilmi[a]**	**Tilva[a]**	**Tia[a]**	**Valne[a]**	**Florf[a]**
MYCAV 176	10	>10	5	8	4	4	>64	5	64	>64	4	0.625	≤0.039	16
MYCAV 177	>10	>10	10	8	4	4	>64	10	>64	>64	4	0.625	≤0.039	32
MYCAV 178	5	>10	10	8	2	4	>64	5	4	>64	0.5	0.312	≤0.039	4
MYCAV 179	10	>10	10	16	4	4	4	0.312	4	4	0.5	1.25	≤0.039	8
MYCAV 180	5	>10	10	>64	4	4	4	0.312	32	>64	1	1.25	≤0.039	8
MYCAV 202	5	>10	5	16	4	4	32	2.5	0.5	0.5	≤0.25	1.25	≤0.039	8

[a]Abbreviations: Enrofl – enrofloxacin; Norfl – norfloxacin; Difl – difloxacin; Spect – spectinomycin; Linko – lincomycin; LiSp – lincomycin-spectinomycin (1:2) combination; Oxy – oxytetracycline; Doxy – doxycycline; Tylo – tylosin; Tilmi – tilmicosin; Tilva – tylvalosin; Tia – tiamulin; Valne – valnemulin; Florf - florfenicol

Among the fluoroquinolones, the MIC values of enrofloxacin and difloxacin showed a wide range (1.25 to >10 µg/ml), while all field isolates had very high MIC values for norfloxacin (≥10 µg/ml) (Figure 6 A, B and C).

The MIC_{50} was 8 µg/ml for spectinomycin and most of the field isolates yielded the MIC_{50} or higher MIC values (Figure 6 D). The MICs for lincomycin clustered around the MIC_{50} value (4 µg/ml) as well, but high MIC values (>64 µg/ml) were yielded in the case of three isolates (Figure 6 E). The MIC_{50} and the MIC_{90} values (4 µg/ml) for lincomycin-spectinomycin (1:2) combination was the same as the MIC_{50} value for lincomycin. In the case of lincomycin-spectinomycin (1:2) combination, the highest concentration needed for inhibition was 32 µg/ml (Figure 6 F).

Broad ranges of the MIC values were observed for tetracyclines (2 to >64 µg/ml for oxytetracycline and 0.078 to >10 µg/ml for doxycycline) with high MIC_{50} and MIC_{90} values (Figures 6 G and H).

Broadest ranges of MIC values were detected for tylosin and tilmicosin (≤0.25 to >64 µg/ml) with high MIC_{50} and MIC_{90} values in the case of tilmicosin (Figures 6 I and J). While the MIC values for tylosin showed diverse distribution, the field isolates' susceptibility profiles formed three groups in the case of tilmicosin (≤0.25, 4 and >64 µg/ml) (Figure 6 J). Among the examined three macrolides (tylosin, tilmicosin and tylvalosin), tylvalosin showed the lowest MIC_{50} value (0.5 µg/ml) against the isolates (Figure 6 K).

From the pleuromutilins the MIC values of tiamulin were higher than those of valnemulin, and the latter compound was found to be the most active antibiotic in the examinations (Figures 6 L and M).

In the case of florfenicol, the susceptibility profiles of most field isolates were similar to each other and showed the MIC_{50} and MIC_{90} value (8 µg/ml) or its two-fold lower dilution (4 µg/ml) with few exceptions (Figure 6 N).

M. anserisalpingitidis samples isolated year by year from the same farms and same tissue types (e.g. MYCAV 34, 53 and 67 from Szentes, phallus lymph; MYCAV 50, 70, 176 and 177 from Cered, phallus lymph; or MYCAV 38 and 202 from Kelebia, cloaca) showed elevated MIC values from year to year in the cases for certain antibiotics. Higher MIC values were detected in subsequent isolates for lincomycin, lincomycin-spectinomycin combination, tetracyclines (both oxytetracycline and doxycycline), macrolides (tylosin, tilmicosin and tylvalosin), tiamulin and for florfenicol as well.

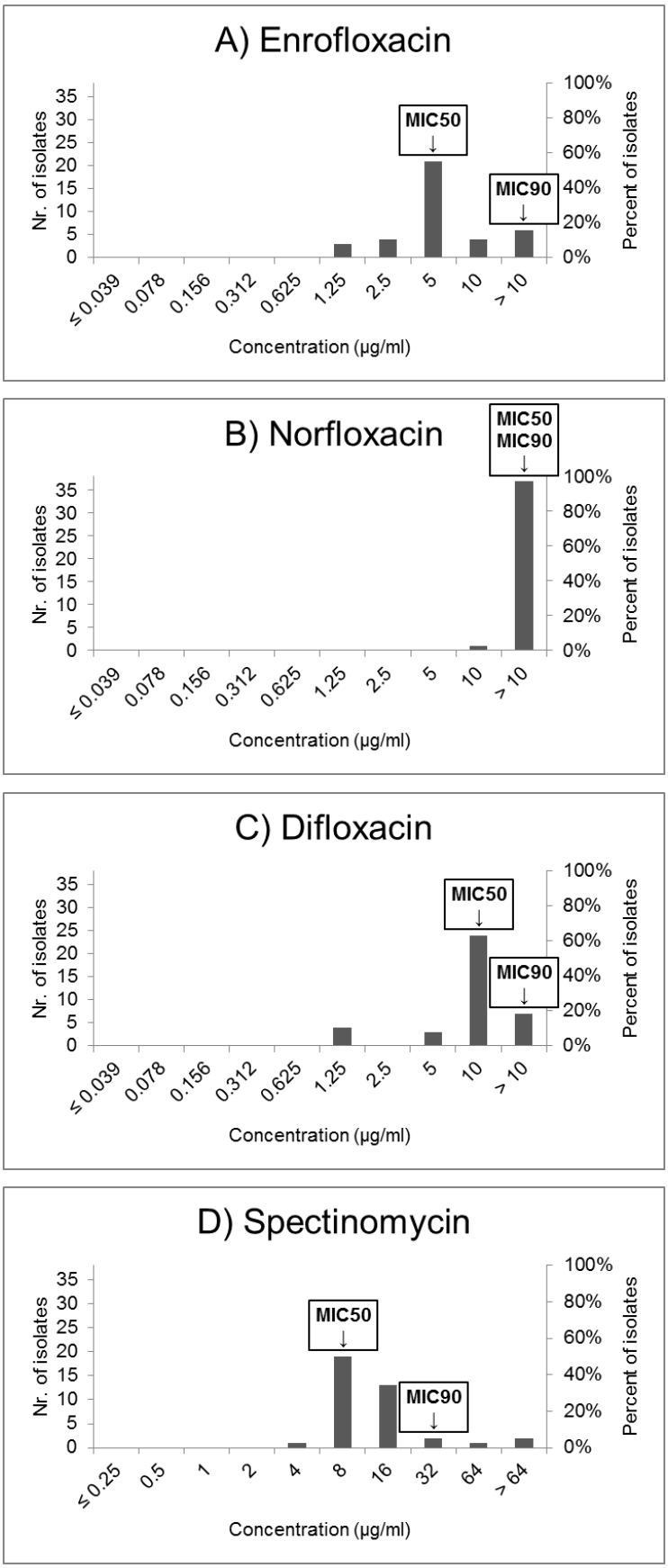

Figure 6. MIC distribution of the tested antibiotics against *M. anserisalpingitidis* isolates.

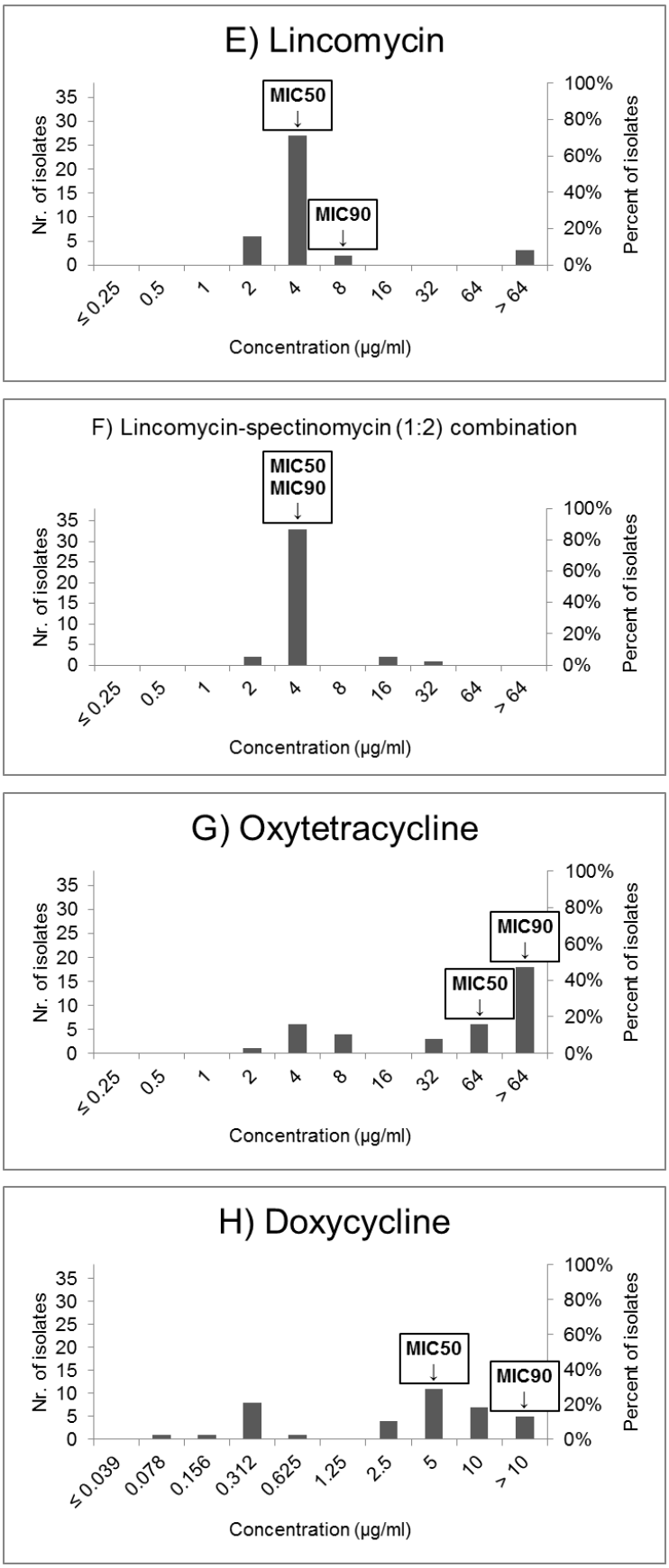

Figure 6 (continued). MIC distribution of the tested antibiotics against *M. anserisalpingitidis* isolates.

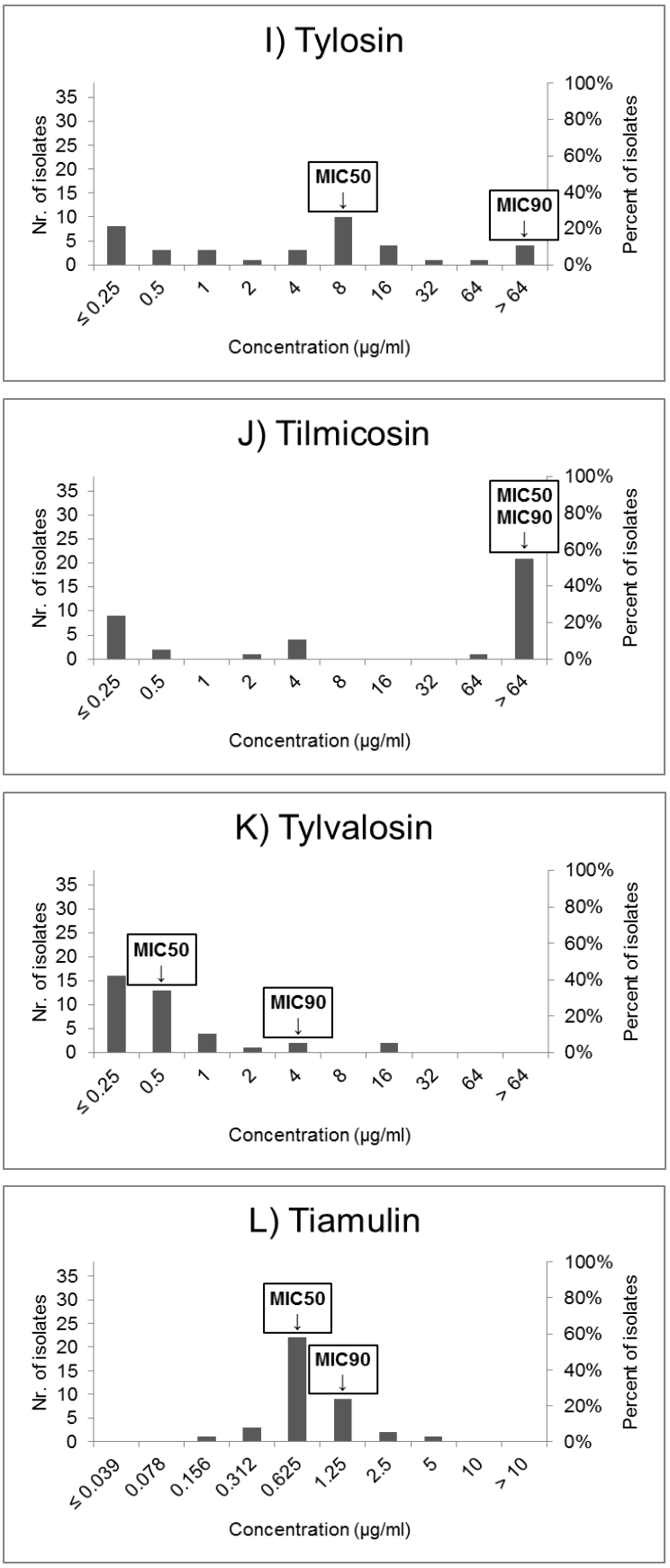

Figure 6 (continued). MIC distribution of the tested antibiotics against *M. anserisalpingitidis* isolates.

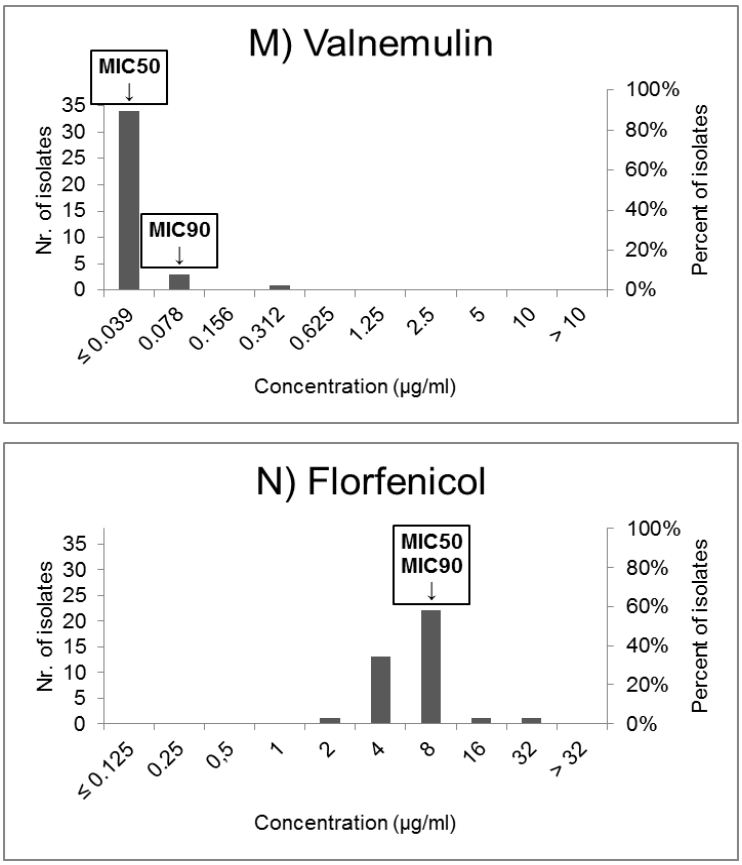

Figure 6 (continued). MIC distribution of the tested antibiotics against *M. anserisalpingitidis* isolates.

5.2. *De novo* genomes of waterfowl *Mycoplasma* strains/isolates

The total genome sizes and information concerning the strains/isolates are detailed in Table 17. The annotated genome sequences were deposited in GenBank, and the raw read data are available in the Sequence Read Archive (SRA). The accession numbers are also listed in Table 17.

The annotated genes with similar features were present at similar percentages in the genomes of the *M. anserisalpingitidis* and *M. anatis* type strains, and likewise in the *M. anseris* and *M. cloacale* type strains (Table 18). Most of the genes (30-38%) had a role during protein metabolism in all four species. Seventeen and 15% of the genes were associated with bacterial carbohydrate metabolism in *M. anserisalpingitidis* and *M. anatis*, respectively, whilst 8% of genes were included in their metabolism in *M. anseris* and *M. cloacale*. The 5-6% of the genes had a role in the amino acid metabolism in the genomes of the *M. anseris* and *M. cloacale* type strains, and only 2% in *M. anserisalpingitidis* and *M. anatis*. Genes associated with microbial respiration were recognised only in the *M. cloacale* genome. In the waterfowl *Mycoplasma* genomes, the following subsystems were listed in the category of cell wall and capsule: lipoprotein sorting system, murein hydrolases, recycling of peptidoglycan amino sugars, and *YjeE*. In the category of virulence, disease and defence DNA gyrase and topoisomerase IV subunit coding genes were listed as possible causatives of fluoroquinolone resistance.

Table 17. Genome information and GenBank accession numbers of waterfowl *Mycoplasma* strains/isolates.

Strain/Isolate	GenBank accession No.	SRA No.	Size (bp)	Total coverage (×)	G+C content (%)	No. of CDS	No. of rRNAs	No. of tRNAs
M. anserisalpingitidis ATCC BAA-2147	CP042295	PRJNA554588	959,110	578	26.7	774	6	32
M. anserisalpingitidis MYCAV 93	CP041663	PRJNA553666	919,993	753	26.7	730	4	32
M. anserisalpingitidis MYCAV 177	CP041664	PRJNA554567	908,787	1288	26.7	742	6	32
M. anatis NCTC 10156	CP030141	SRP155810	956,094	292	26.7	791	6	32
M. anseris ATCC 49234	CP030140	SRP155813	750,009	1,833	26.4	617	6	32
M. cloacale NCTC 10199	CP030103	SRP155814	659,552	1,439	27.0	541	4	31

Table 18. Feature of annotated genes in the genomes of the four waterfowl *Mycoplasma* type strains.

Category of feature	*M. anserisalpingitidis* ATCC BAA-2147 (% of genes)	*M. anatis* NCTC 10156 (% of genes)	*M. anseris* ATCC 49234 (% of genes)	*M. cloacale* NCTC 10199 (% of genes)
Amino acids and derivatives	2	2	5	6
Carbohydrates	17	15	8	8
Cell division and cell cycle	1	1	1	2
Cell wall and capsule	1	1	1	1
Cofactors, vitamins, prosthetic groups, pigments	7	7	4	4
DNA metabolism	12	12	14	13
Fatty acids, lipids, and isoprenoids	3	3	3	3
Membrane transport	5	5	3	4
Nucleosides and nucleotides	2	5	3	3
Potassium metabolism	1	1	1	1
Protein metabolism	31	30	38	31
Respiration	0	0	0	3
RNA metabolism	10	10	12	13
Stress response	3	3	3	3
Sulphur metabolism	1	1	1	1
Virulence, disease and defence	3	3	4	5

Sequence alignment revealed, that the available shotgun projects for the *M. anatis*, *M. anseris* and *M. cloacale* type strains were equivalent to the obtained *de novo* complete genomes.

The whole-genome alignment of the *M. anserisalpingitidis* genomes (Figure 7 A) showed that the type strain and the two field isolates had similar genomic structures, except in one case. A 125-kb inversion (marked with light blue) was revealed in the genome of MYCAV 93 (positions 177,243 to 302,855) surrounded with IS30 family transposase CDSs at both ends of the inversion. In the genome of the ATCC BAA-2147 type strain, IS1634 family transposase CDSs were recognized at both ends of the corresponding genomic region, and the same CDS was found at the 3' end of this region in MYCAV 177.

The whole genome alignment of the type strains of *M. anserisalpingitidis* and *M. anatis* revealed 22 locally collinear blocks, and the size of the longest block was 237 kbp (Figure 7 B). The alignment of the genomes of the *M. anseris* and *M. cloacale* type strains resulted in 84 locally collinear blocks, and the longest block was 92 kbp (Figure 7 C).

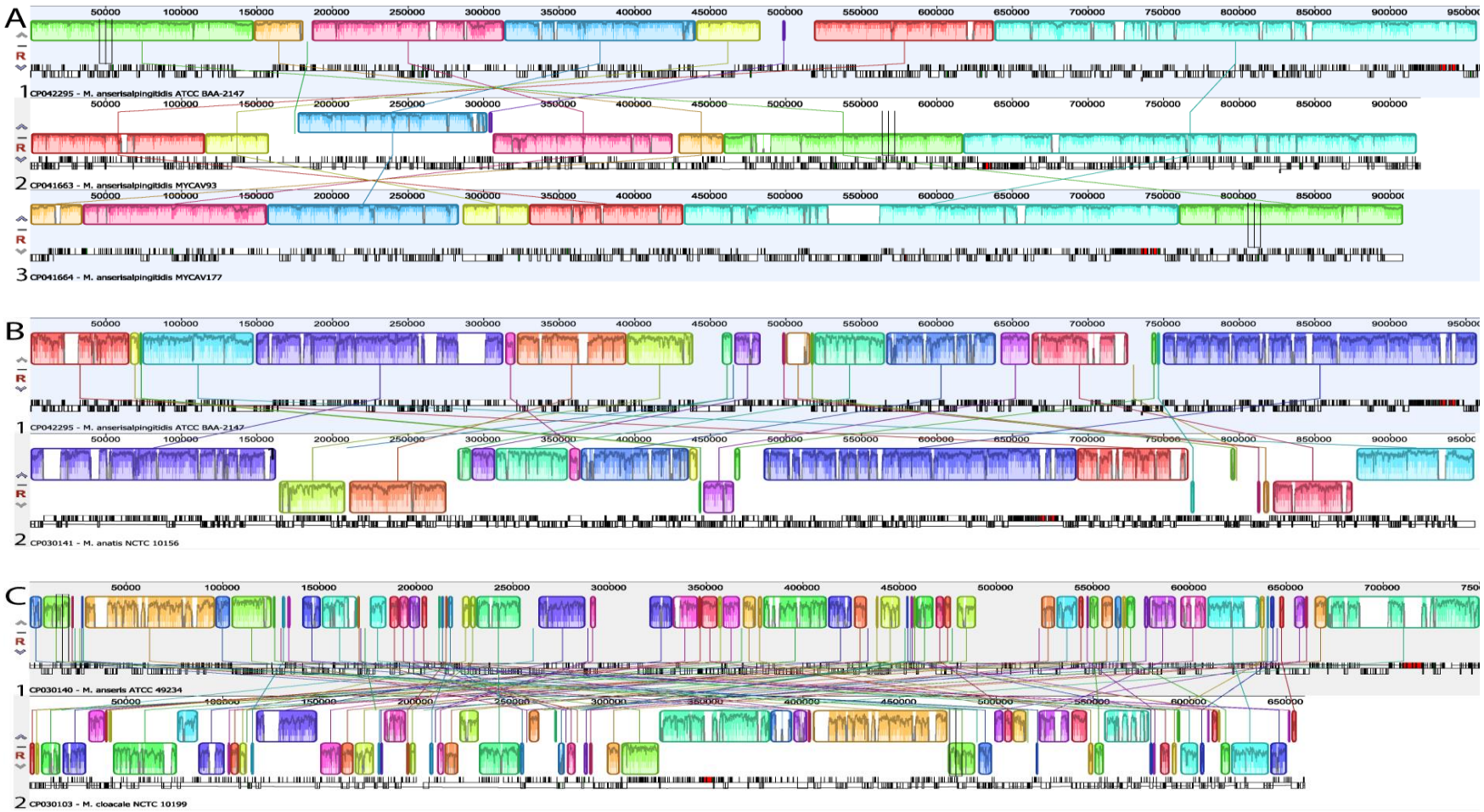

Figure 7. Whole-genome alignment of waterfowl *Mycoplasma* strains/isolates. A: alignment of *M. anserisalpingitidis* ATCC BAA-2147 (1) with *M. anserisalpingitidis* MYCAV 93 (2) and MYCAV 177 (3); B: alignment of *M. anserisalpingitidis* ATCC BAA-2147 (1) with *M. anatis* NCTC 10156 (2); C: alignment of *M. anseris* ATCC 49234 (1) with *M. cloacale* NCTC 10199 (2).
Coloured blocks and lines represent those genomic regions which presumably homologous and internally free from genomic rearrangements. Blocks above or below the centre line suggest the orientation of a genomic region as relative or inverse, compared to the other genome. Regions outside the coloured blocks lack detectable homology among the input genomes.

5.3. Species-specific PCR assays

Sixteen mycoplasma housekeeping genes were analysed in the study, out of which eight genes were found suitable for the design of species-specific primers based on the acceptance criteria. Therefore, the species-specific primers were designed for the following genes: the *pfkB*, *pgmB*, and *rpoB* genes to detect *M. anserisalpingitidis*; the *dnaX*, *pgmB*, *ptsP* and *thyA* genes to detect *M. anatis*; the *dnaX*, *mtn*, and *pcrA* genes to detect *M. anseris*; and the *dnaX* and *mtn* genes to detect *M. cloacale* (Table 10). These primary PCR assays showed species-specificity *in silico*; however, *in vitro* analyses revealed a species cross-amplification in one case (between *M. anatis* and *M. anseris* in the *M. anseris*-specific assay, primer sequences were specific for *dnaX* gene), which was excluded from further testing. The rest of the omitted primers showed a one- to two-fold lower sensitivity in the species-specific PCR amplification, based on UV visualization; hence, they were excluded from further analyses.

According to the combined results on the primers' specificity and sensitivity performed by testing the DNA samples of the *Mycoplasma* type strains, PCR assays, which did not demonstrate any cross-amplification with other tested *Mycoplasma*/*Acholeplasma* strains and showed superior sensitivity, were selected as final assays. The *rpoB* gene-based PCR assay was suitable for precise identification of *M. anserisalpingitidis* (sensitivity of 10^2 GE per reaction), the *dnaX* gene-based PCR assays were the most suitable ones for precise species identification of *M. anatis* and *M. cloacale* (sensitivity of these assays was 10^2 GE per reaction), and the *pcrA* gene-based PCR assay was accepted for species identification of *M. anseris* (sensitivity of 10^1 GE per reaction) (Table 11, Figure 8).

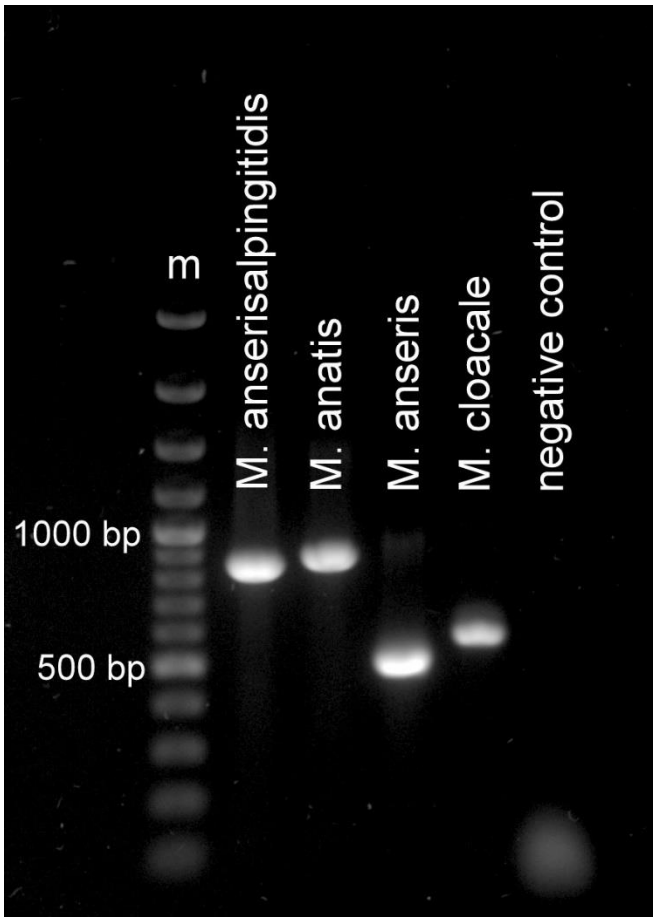

Figure 8. Sizes of the PCR amplicons of *Mycoplasma* species generated by the species-specific PCR assays, with 10^6 GE DNA target.
Abbreviations: m — molecular weight marker (GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific, Waltham, MA, USA)

The similarity between the *dnaX* gene of *M. anatis* and *M. cloacale* is 51.32% and the highest similarity among the bonding sites of the *M. anatis*- and *M. cloacale*-specific primer pairs is 54.55%; therefore, cross-amplification between the two species in the specific PCR assays is unlikely. Species identification was successful in all cases during the tests of the *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale* type strain DNA mixes, the presence of the other waterfowl pathogen mycoplasmas did not have any effect on the assays' performance.

The result of the species-specific PCRs obtained with the testing of 28 clinical specimens is provided in Table 19. Out of the 28 clinical specimens five were negative in all PCR assays, four specimens were positive for one *Mycoplasma* species, nine specimens for two species, other nine specimens for three species and one specimen was positive for all tested *Mycoplasma* species. Sequences of the amplicons obtained from these species-specific assays on the field isolates and clinical specimens (Table 19) showed their 97.7–100.0%

identity with the corresponding sequences of the type strains, confirming that the assays were able to amplify the gene regions specific to the given species.

The *Mycoplasma* genus-specific PCR (Lauerman et al., 1995) amplified PCR products of approximately 460 bp from *M. anserisalpingitidis* and *M. anatis*, and smaller products of approx. 370 bp from *M. anseris* and *M. cloacale*. This assay revealed two amplicons at the corresponding molecular weights when *M. anserisalpingitidis* / *M. anatis* and *M. anseris* / *M. cloacale* co-occurred in the samples, with the exception of three samples. In these three cases (clinical specimens No. 1, 13 and 28, Table 19) a single amplicon was amplified by the genus-specific PCR assay, while the species-specific PCR assays were able to detect mixed infections of *M. anserisalpingitidis*, along with *M. anseris* and/or *M. cloacale* in these geese. Moreover, both *M. anseris* and *M. cloacale* were detected in a clinical specimen (No. 25, Table 19) by the developed assays, which showed the same amplicon size by the genus-specific PCR assay. The DNA sequence chromatogram analysis performed on the genus-specific PCR amplicons from the clinical specimens No. 1 and 25 revealed major sequences of *M. anserisalpingitidis* and *M. anseris*, respectively, with an evidence of well-visible secondary peaks indicating a mixed DNA sequence due to the natural co-infections. However, the DNA sequence chromatogram analysis performed on the genus-specific PCR amplicons from the clinical specimens No. 13 and 28 also revealed major sequences of *M. anserisalpingitidis* and *M. anseris*, respectively, but did not demonstrate any detectable mixed sequences. This discrepancy between the results of the *Mycoplasma* genus-specific PCR and the developed species-specific PCR assays may be associated with a substantial difference of these assays in term of their detection specificity and/or sensitivity for different *Mycoplasma* species, especially if the analysed mycoplasma sample contains mixed DNA.

Table 19. *Mycoplasma* spp. detection in clinical specimens.

No.	Host	Sample source	Country	Place[a]	Year	*Mycoplasma* spp.[b]	*M. anseri-salpingitidis*	*M. anatis*	*M. anseris*	*M. cloacale*
1	goose	cloaca	Hungary	Encsencs	2015	+[g]	+	-	+[e]	+
2	goose	cloaca	Hungary	Szentes	2015	++	+	+[d]	-	+
3	goose	cloaca	Hungary	Encsencs	2016	++	+	+[d]	+	+
4	goose	follicle	Hungary	Tótújfalu	2016	-	-	-	-	-
5	goose	semen	Hungary	Szentes	2016	++	+	+[d]	-	+
6	goose	semen	Hungary	Szentes	2016	++	+	-	-	+[f]
7	goose	semen	Hungary	Szentes	2016	++	+	+[d]	-	+
8	goose	phallus lymph	Hungary	Szentes	2016	-	-	-	-	-
9	goose	trachea	Hungary	Hortobágy	2016	-	-	-	-	-
10	duck	cloaca	Hungary	Kányási and Komádi	2016	-	-	-	-	-
11	duck	phallus lymph	Hungary	Kányási and Komádi	2016	-	-	-	-	-
12	goose	cloaca	Hungary	Érpatak	2017	++	+	-	+	+
13	goose	cloaca	Hungary	Érpatak	2017	+[h]	+	-	+	+
14	goose	cloaca	Hungary	Cered	2017	++	+	-	-	+
15	goose	cloaca	Hungary	Cered	2017	++	+	-	-	+
16	goose	cloaca	Hungary	Derekegyház	2017	++	+	-	+	+
17	goose	cloaca	Hungary	n.d.	2017	++	+	-	-	+
18	goose	cloaca	Hungary	n.d.	2017	++	+	-	-	+
19	goose	cloaca	Hungary	n.d.	2017	++	+	-	+	+
20	goose	cloaca	Hungary	Hajdúsámson	2017	+	-	-	+	-

Table 19 (continued). *Mycoplasma* spp. detection in clinical specimens.

No.	Host	Sample source	Country	Place[a]	Year	*Mycoplasma* spp.[b]	*M. anseri-salpingitidis*	*M. anatis*	*M. anseris*	*M. cloacale*
21	goose	cloaca	Hungary	Hajdúsámson	2017	++	+[c]	-	+	-
22	goose	cloaca	Hungary	Hajdúsámson	2017	+	-	-	+	-
23	goose	cloaca	Hungary	Hajdúsámson	2017	++	+	-	+	-
24	goose	semen	Hungary	Boldogasszonyfa	2017	+	-	-	-	+
25	goose	semen	Hungary	Boldogasszonyfa	2017	+[g]	-	-	+	+
26	goose	cloaca	Hungary	Boldogasszonyfa	2017	++	+	-	+	+
27	goose	cloaca	Hungary	Boldogasszonyfa	2017	+	-	-	-	+
28	goose	phallus lymph	Hungary	n.d.	2018	+[h]	+	-	+	-
					Sum	23	18	4	13	18

[a]Abbreviations: n.d. – no data
[b]+ indicates only one, ++ indicates two PCR products
[c]98.5% amplicon sequence match with the *rpoB* gene of *M. anserisalpingitidis* type strain.
[d]97.8-97.9% amplicon sequence match with the *dnaX* gene of *M. anatis* type strain.
[e]100% amplicon sequence match with the *pcrA* gene of *M. anseris* type strain.
[f]99.5% amplicon sequence match with the *dnaX* gene of *M. cloacale* type strain.
[g]DNA sequence chromatogram indicated mixed infection.
[h]Neither the number of PCR products nor the DNA sequence chromatograms indicated mixed infection.

5.4. MLST study on *M. anserisalpingitidis*

Forty candidate MLST loci were analysed *in silico*, out of which 26 were excluded from further consideration (Table 20). The primers described for the genetic identification of *M. anserisalpingitidis* (Sprygin et al., 2012) (Table 13) have not been further analysed because none of them fulfilled the criteria for locus selection (Table 20). The remaining 14 candidate loci were analysed and evaluated based on Simpson's index of diversity, and the specificity and sensitivity of the PCR assays (Table 20). According to the combined results, the loci of the *atpG*, *fusA*, *pgiB*, *plsY*, and *uvrA* genes were selected finally for the *M. anserisalpingitidis* MLST scheme (Table 20 and Table 14). Based on the genome analyses, all of these genes are present in a single copy in the *M. anserisalpingitidis* genomes. The minimum distance between two MLST loci was 111,940 bp. The sensitivity was 10^3 GE per reaction for all loci. Although the *plsY* locus showed cross-amplification with *M. anatis*, it distinguished a separate clade within the *M. anserisalpingitidis* field isolates showing closer relationship with the genetically very similar *M. anatis* samples (clade A, Figure 9), and was selected among the final loci.

After the expansion of the sequence data with the clinical specimens to altogether 89 *M. anserisalpingitidis* samples, the concatenated sequences of the five loci revealed 76 unique STs with a 0.994 Simpson's index of diversity. Allele numbers, and sequence types for all investigated *M. anserisalpingitidis* samples are described in Table 21. A 'PubMLST' database was set up for this MLST scheme for *M. anserisalpingitidis* (https://pubmlst.org/manserisalpingitidis/). The number of unique alleles, total single nucleotide polymorphisms (SNPs), non-synonymous SNPs, and Simpson's index of diversity for each locus is summarised in Table 22. Phylogenetic tree constructed from the concatenated nucleotide sequences of all five loci of *M. anserisalpingitidis* samples showed highly congruent topology with the samples' background data and with the published cgMLST scheme (Kovács et al., 2020), and revealed three clades (clade A-C), and six subclades within clade C (Figure 9).

Table 20. List of the genes examined during the development of the MLST scheme for *M. anserisalpingitidis*.

No.	Gene	Results	Simpson's index of diversity of the internal fragment calculated for the 82 *M. anserisalpingitidis* genomes
1	*atpG*	10^3 GE sensitivity	0.962
2	*fusA*	10^3 GE sensitivity	0.893
3	*pgiB*	10^3 GE sensitivity	0.930
4	*plsY*	10^3 GE sensitivity, cross-amplification with *M. anatis*	0.952
5	*uvrA*	10^3 GE sensitivity	0.921
6	*dnaA*	10^4 GE sensitivity	0.940
7	*efp*	10^3 GE sensitivity, genetic distance to *pgiB*, lower diversity	0.927
8	*gmk*	10^3 GE sensitivity, genetic distance to *pgiB*, lower diversity	0.900
9	*leuS*	10^3 GE sensitivity	0.862
10	*pyrH*	10^1 GE sensitivity, cross-amplification with *M. anatis*	0.905
11	*rpoB*	10^2 GE sensitivity, good sensitivity, lower diversity	0.877
12	*ruvB*	10^3 GE sensitivity, genetic distance from *fusA*, lower diversity	0.876
13	*tpiA*	10^3 GE sensitivity, cross-amplification with *M. anatis*	0.888
14	*metG*	insufficent amplification	0.911
15	*ugpA*	gene present in multiple copies	
16	*ackA*	low diversity or unsuitable for primer design	
17	*adk*	low diversity or unsuitable for primer design	
18	*alaS*	low diversity or unsuitable for primer design	
19	*algA*	low diversity or unsuitable for primer design	
20	*argS*	low diversity or unsuitable for primer design	
21	*aspS*	low diversity or unsuitable for primer design	
22	*calB*	low diversity or unsuitable for primer design	
23	*dnk*	low diversity or unsuitable for primer design	
24	*gltX*	low diversity or unsuitable for primer design	
25	*gyrB*	low diversity or unsuitable for primer design	
26	*lepA*	low diversity or unsuitable for primer design	
27	*pfkA*	low diversity or unsuitable for primer design	
28	*ppa*	low diversity or unsuitable for primer design	
29	*recA*	low diversity or unsuitable for primer design	
30	*rnr*	low diversity or unsuitable for primer design	
31	*rpoC*	low diversity or unsuitable for primer design	
32	*tufA*	low diversity or unsuitable for primer design	
33	*valS*	low diversity or unsuitable for primer design	
34	*pfkB*	not present in all genomes	
35	*dppC*	not present in the type strain's genome	
36	*kdpA*	not present in the type strain's genome	
37	*metS*	not present in the type strain's genome	
38	*nagC*	not present in the type strain's genome	
39	*nanA*	not present in the type strain's genome	
40	*ulaA*	not present in the type strain's genome	
41	*dnaE*	high variability on the primer-specific region, high PCR product size	0.980
42	*fusA*	high PCR product size	0.924
43	*pyk*	high variability on the primer-specific region, high PCR product size	0.949
44	*rpoB*	low diversity	0.687

Table 21. MLST typing result of the *89 M. anserisalpingitidis* samples.

No.	Sample ID	Allelic No.					Sequence type
		atpG	***fusA***	***pgiB***	***plsY***	***uvrA***	
1	ATCC BAA-2147	1	1	1	1	1	1
2	MYCAV 34	2	2	2	2	2	2
3	MYCAV 38	3	3	3	3	3	10
4	MYCAV 39	4	4	4	4	4	16
5	MYCAV 40	5	5	5	5	3	17
6	MYCAV 44	6	6	6	6	5	18
7	MYCAV 47	3	2	2	7	2	7
8	MYCAV 49	2	2	5	8	6	4
9	MYCAV 50	2	2	2	8	2	3
10	MYCAV 51	3	2	5	8	2	8
11	MYCAV 53	7	2	5	9	2	19
12	MYCAV 54	7	2	5	9	2	19
13	MYCAV 55	8	5	6	10	4	29
14	MYCAV 56	9	7	7	11	7	30
15	MYCAV 61	10	8	8	12	8	31
16	MYCAV 63	9	7	7	11	7	30
17	MYCAV 65	2	5	9	13	9	5
18	MYCAV 66	11	9	10	9	3	32
19	MYCAV 67	12	10	5	9	10	33
20	MYCAV 68	7	10	11	9	10	27
21	MYCAV 69	13	5	2	2	11	34
22	MYCAV 70	3	2	12	8	2	9
23	MYCAV 71	9	7	7	11	7	30
24	MYCAV 72	14	6	6	14	12	35
25	MYCAV 73	9	7	7	11	7	30
26	MYCAV 75	15	11	9	15	3	36
27	MYCAV 76	16	12	11	16	13	38
28	MYCAV 77	9	7	7	11	7	30
29	MYCAV 78	9	7	7	11	7	30
30	MYCAV 91	17	2	13	17	14	39
31	MYCAV 93	18	13	14	18	15	40
32	MYCAV 94	2	12	15	19	16	6
33	MYCAV 177	7	2	12	8	10	20
34	MYCAV 178	19	5	2	2	2	41
35	MYCAV 179	20	14	16	4	17	42
36	MYCAV 180	16	6	17	15	3	37
37	MYCAV 202	21	3	18	20	3	43
38	MYCAV 205	22	5	2	2	2	44
39	MYCAV 212	7	10	5	8	18	26
40	MYCAV 218	7	10	5	8	2	25
41	MYCAV 221	23	10	2	21	6	45
42	MYCAV 222	24	2	2	2	19	46
43	MYCAV 243	25	10	11	22	20	47
44	MYCAV 245	26	10	2	8	2	48
45	MYCAV 247	4	2	2	23	2	15
46	MYCAV 248	27	2	2	23	2	49
47	MYCAV 264	3	5	12	24	10	11
48	MYCAV 270	7	2	12	25	2	23
49	MYCAV 271	7	2	12	8	19	21
50	MYCAV 275	28	15	2	26	21	50
51	MYCAV 313	29	10	19	8	22	51
52	MYCAV 325	30	2	2	27	23	52
53	MYCAV 327	31	10	11	28	2	54
54	MYCAV 332	32	10	5	28	6	55

Table 21 (continued). MLST typing result of the *89 M. anserisalpingitidis* samples.

No.	Sample ID	Allelic No.					Sequence type
		atpG	***fusA***	***pgiB***	***plsY***	***uvrA***	
55	MYCAV 333	3	16	5	28	2	14
56	MYCAV 340	33	12	13	29	24	56
57	MYCAV 342	34	10	11	28	25	57
58	MYCAV 343	7	2	12	28	6	24
59	MYCAV 382	35	6	20	29	26	58
60	MYCAV 423	7	2	12	9	10	22
61	MYCAV 424	3	10	21	8	2	12
62	MYCAV 429	7	2	12	9	10	22
63	MYCAV 430	30	10	12	8	2	53
64	MYCAV 449	36	10	11	28	6	59
65	MYCAV 452	37	5	22	30	27	60
66	MYCAV 493	38	17	13	31	3	62
67	MYCAV 494	38	12	13	31	24	61
68	MYCAV 498	3	10	21	8	25	13
69	MYCAV 500	31	10	11	28	2	54
70	MYCAV 502	7	18	5	28	6	28
71	MYCAV 512	39	8	2	17	2	63
72	MYCAV 665	40	19	23	32	28	64
73	MYCAV 666	41	20	24	33	29	65
74	MYCAV 667	42	21	25	34	30	67
75	MYCAV 668	42	21	25	34	30	67
76	MYCAV 669	43	22	26	35	31	68
77	MYCAV 670	44	19	27	36	32	69
78	MYCAV 675	45	23	28	37	33	70
79	MYCAV 680	41	20	29	33	34	66
80	MYCAV 783	46	24	30	38	35	71
81	MYCAV 785	46	24	30	38	36	72
82	MYCAV 903	47	8	31	39	37	73
83	PL 1	44	19	27	36	32	69
84	PL 2	44	19	27	36	32	69
85	PL 3	48	25	32	40	38	74
86	PL 5	40	19	23	32	28	64
87	PL 11	49	20	29	33	34	75
88	PL 13	41	20	24	33	29	65
89	UA 1	50	26	2	41	4	76

Table 22. The number of alleles, SNPs, and Simpson's index of diversity for each locus and for the MLST scheme based on the sequence analysis of 89 *M. anserisalpingitidis* samples.

Locus	No. of allele types	Total number of SNPs/length of the examined locus (bp)	No. of non-synonymous SNPs/length of the examined locus (bp)	Simpson's index of diversity
atpG	50	62/450 (13.78%)	15/450 (3.33%)	0.966
fusA	26	74/577 (12.82%)	15/577 (2.60%)	0.906
pgiB	32	33/284 (11.62%)	9/284 (3.17%)	0.936
plsY	41	84/325 (25.85%)	22/325 (6.77%)	0.957
uvrA	38	60/373 (16.09%)	13/373 (3.49%)	0.931
MLST scheme	76 ST[a]	313/2009 (15.58%)	74/2009 (3.68%)	0.994

[a]Sequence type

Figure 9.
Caption in the next page

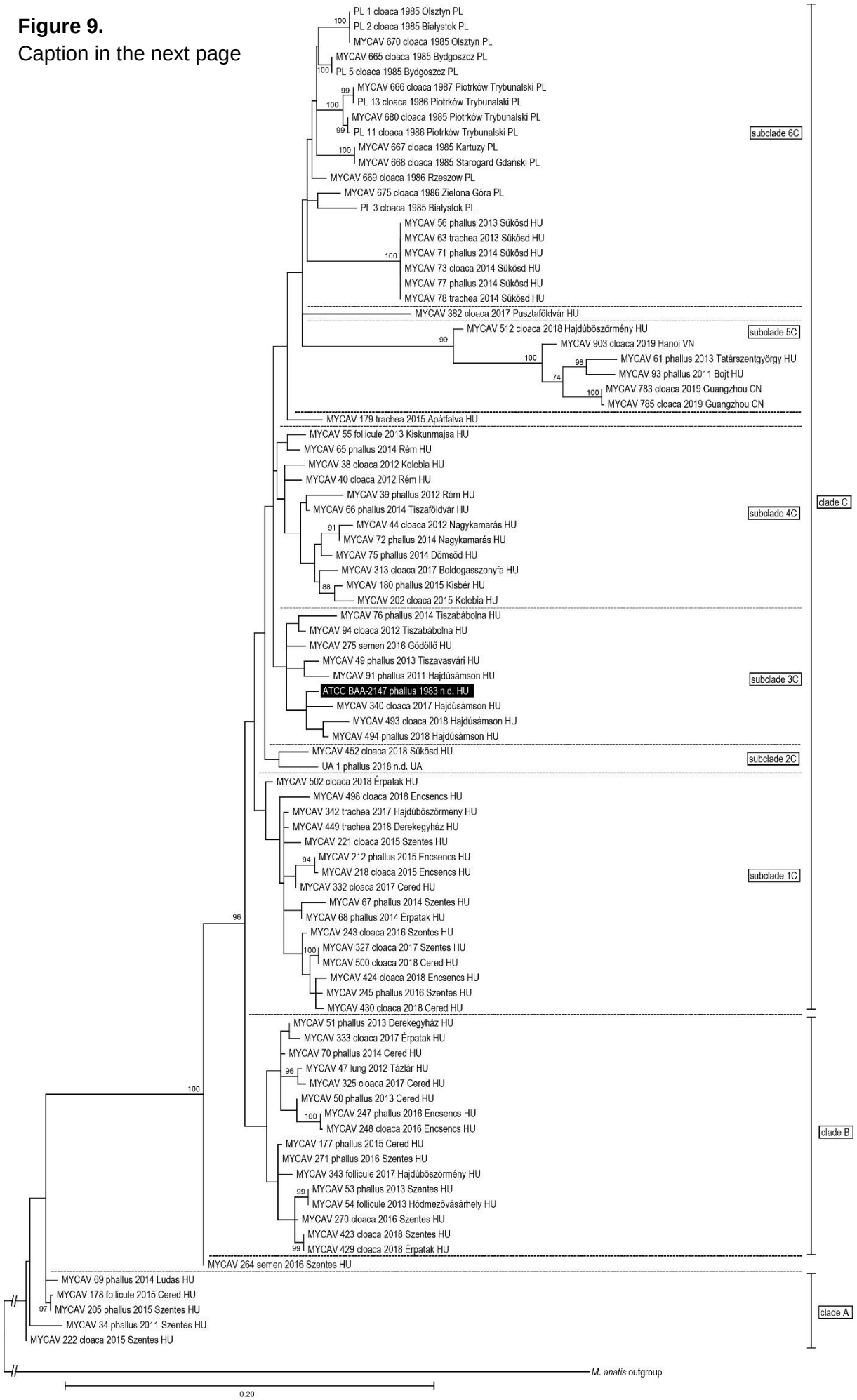

Figure 9. Maximum Likelihood phylogenetic tree showing the relationship between the 89 investigated *M. anserisalpingitidis* samples and *M. anatis* outgroup based on MLST analyses.

Major clades of *M. anserisalpingitidis* are dedicated as clade A, B, and C, and subclades 1-6C. Countries are indicated as follows: CN – China, HU – Hungary, PL – Poland, UA – Ukraine, VN – Vietnam. Abbreviations: n.d. – no data. The *M. anserisalpingitidis* ATCC BAA-2147 type strain is highlighted in black. Bootstrap values of neighbour joining (1000 replicates) of >70 are shown. The scale bar represents the average number of substitutions per site.

Five Hungarian isolates were included in clade A. The sequence analysis revealed that these field isolates showed 100% sequence similarity with the *M. anatis* samples for the *plsY* locus, whilst sequence similarity was 82% to *M. anserisalpingitidis* ATCC-BAA 2147 type strain. In the phylogenetic tree, these five *M. anserisalpingitidis* samples were located closest to the *M. anatis* outgroup. Clade B contained sixteen Hungarian isolates mostly from the Eastern part of Hungary, including the sole *M. anserisalpingitidis* isolate originating from duck (MYCAV 47; ST-7). Many Hungarian isolates and all samples originating from the other examined countries were sorted into clade C. Subclade 1C comprised sixteen Hungarian isolates, mostly from Eastern Hungary. The Ukrainian sample (UA 1; ST-76) and a Hungarian isolate formed subclade 2C, their concatenated sequences showed 98% similarity. The ATCC BAA-2147 type strain (ST-1) was placed into subclade 3C together with mostly Eastern Hungarian field isolates. Subclade 4C comprised field isolates originating from several parts of Hungary. Subclade 5C comprised three Hungarian (MYCAV 61, 93, and 512; ST-31, -40, and -63, respectively), two Chinese (MYCAV 783, and 785; ST-71, and -72, respectively) and a Vietnamese (MYCAV 903; ST-73) *M. anserisalpingitidis* isolates, their sequence similarities were in the range of 97-100%. In the phylogenetic tree, this subclade showed the highest genetic distance among the subclades of clade C. Subclade 6C contained all fourteen Polish samples, and a Hungarian ST (ST-30; n=6, isolated from the same farm in two consecutive years). Sequence similarity was 97-98% on the concatenated sequences between the Polish samples and the Hungarian ST. Three Hungarian isolates (MYCAV 179, 264, and 382; ST-42, -11, and -58, respectively) were defined as outliers.

The examined Hungarian livestock integration's samples (Table 1) were divided into clade A (n=4), clade B (n=15), and subclade 1C (n=16), while one isolate did not cluster with any clades (MYCAV 264). The 36 samples yielded 33 different STs. In the *M. anserisalpingitidis* sample collection, all field isolates from the same flock or the same animal's different organs (Table 1) had different STs.

6. Discussion

6.1. Antibiotic susceptibility profile of *M. anserisalpingitidis*

Before our study, information about the susceptibility of *M. anserisalpingitidis* field isolates to antimicrobials was scarce, as only one paper revealed data concerning the antibiotic susceptibility of this species (Stipkovits and Szathmary, 2012). In that paper, the authors determined the values of enrofloxacin, tylosin, chlortetracycline, oxytetracycline, doxycycline, tiamulin and lincomycin in *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale*, although detailed data on their method is lacking (Stipkovits and Szathmary, 2012). Thus, we were facing the absence of reports about the antibiotic susceptibility of *M. anserisalpingitidis* and also of other *Mycoplasma* species occurring in waterfowl. Therefore, the results of the antibiotic susceptibility of the concerned species (Table 15 and 16) were also compared to data of antibiotic susceptibility of the well-studied *Mycoplasma* species of poultry: *M. synoviae* and *M. gallisepticum*, gathered until 2016.

Elevated MIC values were reported previously in the case of the fluoroquinolones, especially of enrofloxacin in *M. anserisalpingitidis* (MIC_{50} 2 µg/ml and MIC_{90} 4 µg/ml) and other *Mycoplasma* species of poultry (Gautier-Bouchardon et al., 2002; Landman et al., 2008; Stipkovits and Szathmary, 2012; Lysnyansky et al., 2013). The increasing occurrence of quinolone-resistant *M. synoviae* and *M. gallisepticum* field isolates were also observed (Gerchman et al., 2008; Landman et al., 2008). In the current study, the detected MIC_{50} values (5 µg/ml for enrofloxacin, 10 µg/ml for difloxacin and ≥10 µg/ml for norfloxacin) were even higher than the ones reported before (Gautier-Bouchardon et al., 2002; Gerchman et al., 2008; Landman et al., 2008; Stipkovits and Szathmary, 2012; Lysnyansky et al., 2013), confirming the observation of increasing quinolone-resistance in *Mycoplasma* species. In order to save these antibiotics for human disease treatment the directive was to reduce the use of these agents in livestock. Former efforts to prevent the appearance of quinolone-resistant species were proved to be unsuccessful considering the observed dramatic elevations in the MIC values of these antibiotics in avian *Mycoplasma* species (European Medicines Agency, 2010; Gautier-Bouchardon et al., 2002; Landman et al., 2008).

The administration of the combination of lincomycin and spectinomycin reduced the number of the diseased ganders and egg infertility rates, and increase the hatching rates and the egg production in *M. anserisalpingitidis* infected geese (Czifra et al., 1986). The lincomycin-spectinomycin therapy was proved to be effective against other *Mycoplasma* species as well; however, application of spectinomycin in monotherapy is not recommended due to its insufficient effectiveness and relatively high MIC values in *in vitro* experiments (Behbahan et al., 2008). *In vitro* effectiveness of lincomycin at 2 µg/ml MIC_{50} values against

M. anserisalpingitidis, *M. anatis* and *M. anseris* species has been reported (Stipkovits and Szathmary, 2012). In the present study, all field isolates showed elevated MIC values for spectinomycin, lincomycin and lincomycin-spectinomycin combination. The growth of a couple of isolates was not inhibited even at the highest concentrations used (64 µg/ml) for spectinomycin and lincomycin individually. The combination of the two antibiotics improved their effectiveness, as lincomycin-spectinomycin combination could inhibit the growth of all examined field isolates within the concentration range used (0.25 to 64 µg/ml) and a lower MIC_{90} value was observed too.

Previously, tetracyclines (chlortetracycline, doxycycline and oxytetracycline) showed 1–2 µg/ml MIC_{50} values against *M. anserisalpingitidis* isolates. Growth of other *Mycoplasma* species isolated from waterfowl were inhibited at 2–4 µg/ml MIC_{50} values using the same antibiotics (Stipkovits and Szathmary, 2012). *Mycoplasma* species infecting poultry were observed to be inhibited by elevated MIC values, although with exceptions, as *M. synoviae* strains showed high susceptibility to doxycycline in The Netherlands (Wang et al., 2001; Behbahan et al., 2008; Landman et al., 2008). In the current study, different concentration ranges were used for oxytetracycline and doxycycline because of the suspected MIC values based on previous studies with *M. synoviae* and *M. gallisepticum* (Wang et al., 2001; Gautier-Bouchardon et al., 2002; Behbahan et al., 2008; Landman et al., 2008; Gharaibeh and Al-Rashdan, 2011). Although the *M. anserisalpingitidis* field isolates showed broad ranges of MIC values for oxytetracycline and doxycycline, more than 50% of the isolates were inhibited by only higher antibiotic concentrations (64 and 5 µg/ml, respectively) and MIC_{90} values exceeded the concentration ranges used for both compounds. These results show a dramatic increase of the MIC values of tetracyclines against *M. anserisalpingitidis* isolates and reveals the presence of probably highly resistant field isolates in Hungary.

Macrolides, especially tylvalosin have good *in vitro* effectiveness against most *Mycoplasma* species infecting poultry, showing lower MIC values in previous examinations than quinolones and tetracyclines (Wang et al., 2001; Behbahan et al., 2008; Landman et al., 2008; Forrester et al., 2011; Stipkovits and Szathmary, 2012). However, *M. gallisepticum* could develop resistance rapidly to these compounds, especially to tilmicosin (Wu et al., 2005). Earlier, the MIC_{50} values in *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* isolates were defined to be 2 µg/ml for tylosin (Stipkovits and Szathmary, 2012). In the current study, the MIC_{50} value (8 µg/ ml) of tylosin was higher than the previous observation (Stipkovits and Szathmary, 2012), and the MIC_{90} value exceeded the concentration range used in the experiment. However, high variability was observed in the susceptibility of the field isolates to this compound. In the practice, tylosin was used successfully for the treatment of mycoplasmosis in geese (Czifra et al., 1986). Similarly, wide range of the MIC values was detected for tilmicosin, highlighting the necessity of susceptibility testing before the antibiotic

treatments. As opposed to the diverse susceptibility profiles of the isolates for tylosin, the MIC values of tilmicosin were categorized into three separate groups. The observed distribution of the MIC values is likely in association with the capability of *M. anserisalpingitidis* to develop resistance more rapidly to tilmicosin (i.e. with one or two mutations) than to other macrolides. The same phenomenon was described in other *Mycoplasma* species as well (Wu et al., 2005). Out of the three macrolides examined in the study, tylvalosin was the most effective agent against *M. anserisalpingitidis* field isolates, showing a lower MIC_{50} value (0.5 µg/ml) against the pathogen than the majority of the antibiotics tested.

Pleuromutilins showed good *in vitro* effectiveness against avian *Mycoplasma* species in previous studies and low tendency of the development of resistance to these agents has been reported (Drews et al., 1975; Gautier-Bouchardon et al., 2002; Li et al., 2010; Xiao et al., 2015). Tiamulin was used successfully for the treatment of mycoplasmosis and its effectiveness was similar to lincomycin-spectinomycin therapy in the treated geese (Czifra et al., 1986). Low MIC values (MIC_{50}: 0.06 µg/ml, MIC_{90}: 0.25 µg/ml) of tiamulin were described in the case of *M. anserisalpingitidis*, and similarly low MIC_{50} values (0.125–1 µg/ml) were observed against *M. anatis*, *M. anseris* and *M. cloacale* (Stipkovits and Szathmary, 2012). In the present study, pleuromutilins were found to be the most effective antibiotic agents among the examined compounds, especially valnemulin showed high *in vitro* effectiveness against all tested isolates of the pathogen. However, it is noteworthy, that field isolates with elevated MIC values were detected for tiamulin (MIC: 2.5–5 µg/ml) and even for valnemulin (MIC: 0.312 µg/ml). Although the low MIC values of valnemulin against *M. anserisalpingitidis* isolates *in vitro* are promising for its clinical use, it should be noted that in a previous study only a single mutation in *M. gallisepticum* could cause elevation in the MIC values of valnemulin (Li et al., 2010). To date, the use of pleuromutilins in humans is limited, as there are only two commercially available products authorized containing this active substance. However, bacterial strains resistant to pleuromutilins have already been described and these strains also show a multidrug resistance, which warrants the prudent use of these antibiotic agents (van Duijkeren et al., 2014; FDA, 2019).

Phenicols showed a good *in vitro* activity against *Mycoplasma* species of poultry, but information about their effectiveness in waterfowl is lacking (Lin, 1987; Gharaibeh and Al-Rashdan, 2011). In the present study, most of the *M. anserisalpingitidis* field isolates yielded the same MIC values (4 or 8 µg/ml) for florfenicol, and only two isolates (originating from the same region) showed elevated MIC values compared to the MIC_{50} (8 µg/ml), one of them reaching the highest antibiotic concentration (32 µg/ml) used.

The elevated MIC values of several antibiotics detected in subsequent isolates from the same farms from year to year are likely in association with the inconsistent use of antibiotics and the rapid development of antibiotic resistance. The *M. anserisalpingitidis* type strain with

unknown exact location was isolated almost 30 years before the examined field isolates; however, we did not find any differencies in the antibiotic susceptibility profile of this strain compaired to the recent isolates. Nevertheless, as enrofloxacin, difloxacin, and the three macrolides proved to be effective against this strain, a continuous follow-up study could have provided valuable data about the development of antibiotic resistance in *M. anserisalpingitidis*.

Valnemulin, tiamulin and tylvalosin were found to be the most effective antibiotics in the present study. Most of the field isolates showed elevated MIC values for more than one agent, but none of the isolates yielded high MIC values for all the examined antibiotics. Nevertheless, the results confirmed that increasing resistance can be observed in the case of several antibiotics. These findings highlight the consistent use of antibiotics and the need for the determination of antibiotic susceptibility of *Mycoplasma* species prior to applying the treatment.

6.2. *De novo* genomes of waterfowl *Mycoplasma* strains/isolates

Comprehensive molecular biology and bioinformatics research require the knowledge of the genetic background of the studied organism. At the time of our study, only gene fragments of *M. anserisalpingitidis* were available in the GenBank. For *M. anatis*, a shotgun sequencing project was reported (Guo et al., 2011), and unpublished sequences from shotgun projects as well were available for the *M. anseris* and *M. cloacale* type strains. However, these shotgun projects contain many scaffolds (e.g. the *M. anatis* project consists of 44 records) and the length of the scaffolds highly varies (from 1,000 to 148,000 bp). Therefore, the handling of these sequences is complicated. As a result of the performed *de novo* sequence projects easily accessible complete genomes have been created suitable for the analyses on basic sequence analysing platforms, commonly available for scientists worldwide.

The size and the low G+C content of the genomes of the *M. anserisalpingitidis*, *M. anatis*, *M. anseris*, and *M. cloacale* type strains was also characteristic to other mycoplasmas (Sirand-Pugnet et al., 2007; Kleven, 2008). Among the four complete genomes, the *M. anatis* type strain had the highest number of CDSs, despite that the *M. anserisalpingitidis* type strain was slightly longer. The genome of the *M. cloacale* type strain contained the lowest number of CDSs out of the the four *de novo* genomes in accordance with its genome size (Table 17).

Almost twice as much genes were associated with carbohydrates in the complete genomes of *M. anserisalpingitidis* and *M. anatis* type strains than in *M. anseris* and *M. cloacale*. In contrast, the genomes of *M. anseris* and *M. cloacale* contained more genes for amino acid metabolisms than *M. anserisalpingitidis* and *M. anatis* (Table 18). These observations are in accordance with the glucose-splitting metabolism of *M. anserisalpingitidis* and *M. anatis*, and

with the arginine utilization of *M. anseris* and *M. cloacale* (Stipkovits et al., 1984b; Kleven, 2006, 2008). Interestingly, some genes were listed in the category of cell wall and capsule despite mycoplasmas lack the cell wall; however, a few genes associated with lipoproteins were also sorted into this category. Nevertheless, Table 18 was based on RAST annotation, whilst most of the listed genes in this category were not found by the PGAP software. It could be some discrepancy between the two systems or these genes could also be functionless in the genomes. Mutations in the listed gyrase and topoisomerase IV coding genes have already been associated with fluoroquinolone resistance in mycoplasmas (Reinhardt et al., 2002; Lysnyansky et al., 2013; Bekő et al., 2020) and the observed resistance to fluoroquinolones in waterfowl pathogen *Mycoplasma* species was described in the present thesis (section 6.1). The MIC values against high number of field isolates and genome sequences may help to reveal the mutations associated with antibiotic resistance, and the complete genomes of the waterfowl *Mycoplasma* type strains provide a basis for exploring the genetic basis of antibiotic resistance mechanisms.

As *M. anserisalpingitidis* was in the focus of our research, two field isolates with different origins were sequenced in addition to the type strain. The comparison of the three genomes revealed an inversion (Figure 7 A); similarly to previously described genomic rearrangement in other *Mycoplasma* species (Vasconcelos et al., 2005; Li et al., 2011). The products of the IS30 and IS1634 family transposase CDSs which were found at both ends of the corresponding regions could be involved in chromosomal rearrangements or contribute to horizontal gene transfer (Loreto et al., 2007). Different numbers of CDSs and rRNAs were detected among the three strains which is concerned another frequently experienced genetic characteristic in mycoplasmas (Li et al., 2011; Wise et al., 2011).

The waterfowl *Mycoplasma* type strains' genomes were compared with each other considering the species pairs' high similarity on the 16S rRNA, that they belong to the Synoviae or Hominis cluster of mycoplasmas, respectively (Volokhov et al., 2012, 2020), and because of the observed similar gene repertoire (Table 18). The observed high coverage between the genomes of the *M. anserisalpingitidis* and *M. anatis* type strains (Figure 7 B) supports the theory that these mycoplasmas may have separated from a common ancestor and they are closely related (Volokhov et al., 2020). The locally collinear blocks also covered the input genomes of *M. anseris* and *M. cloacale* and the outside blocks were inconsiderable (Figure 7 C), indicating that these species also share highly homologous regions of genomes. However, the high number of short blocks and their intense rearrangements also suggest that these species are more distinct and separated from each other than *M. anserisalpingitidis* and *M. anatis*. Nevertheless, a more complex genome comparison including several isolates of the investigated species could provide a better understanding of the relationship between different avian *Mycoplasma* species.

The *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* type strains' genomes were essential for performing the waterfowl *Mycoplasma* species-specific PCR, and the *M. anserisalpingitidis* MLST studies. Hence, these publicly available records could be the basis for further scientific projects with worldwide relevance and interest.

6.3. Species-specific PCR assays

Co-infection of waterfowl with *M. anserisalpingitidis*, *M. anatis*, *M. anseris* and *M. cloacale* that share similar phenotypic characteristics is frequent, and therefore the isolation of pure culture and the precise identification of these species by biochemical and serological tests are challenging issues in routine veterinary laboratories. Species-specific PCR assays could improve the specific detection of these mycoplasmas in clinical specimens from geese and ducks.

The nucleotide sequences of the 16S rRNA genes of *M. anserisalpingitidis* and *M. anatis*, along with *M. anseris* and *M. cloacale* demonstrate significant similarities (99.0% and 98.3%, respectively) and therefore cannot be used for the unambiguous differentiation these species pairs. Also, the high percentage of the 16S-23S rRNA ITS sequences (97.4% and 87.3%, respectively) makes these genetic markers less suitable for a precise identification of these species (Volokhov et al., 2012, 2020). Previously published PCR assays targeting these genetic regions for the detection of mycoplasmas (Lauerman et al., 1995; Ramírez et al., 2008; Raviv and Kleven, 2009; Pisal et al., 2016) demonstrated a low reliability in the identification of waterfowl *Mycoplasma* species. Moreover, these methods almost always require the direct sequencing of amplicons for the species confirmation, which can be difficult or impossible in the case of co-infections of multiple *Mycoplasma* species, especially in those laboratories in which DNA sequencing technologies are not available.

The *Mycoplasma* genus-specific PCR assay (Lauerman et al., 1995) provides different amplicon sizes for certain *Mycoplasma* species (e.g. 90 bp difference between amplicon sizes of *M. anserisalpingitidis* / *M. anatis* and *M. anseris* / *M. cloacale*) due to the length variation of the 16S-23S rRNA ITS among the species (Volokhov et al., 2012). Therefore, amplicons at different molecule weights already indicate co-occurrence of *Mycoplasma* species. However, the detection of only one amplicon does not necessarily means the presence of a single species, and in certain cases even the analysis of the chromatograms can not indicate the presence of co-occurring species. In the current study, the comparison of the results of primary screening of clinical samples using the genus-specific PCR assay with the results of the developed species-specific PCR assays revealed discrepancies in certain cases (Table 19). The observed differences may be the consequences in the ratio between the mixed DNAs presented in the clinical samples, which can influence their equally efficient detectability

(Kobelt et al., 1998). The presented results confirm that the 16S-23S ITS-based PCR assay is well suitable for the primary detection or screening of clinical specimens for the presence of mycoplasma DNA; however, it is unsuitable for the detection and identification of all presented species in samples from mixed *Mycoplasma* infection cases. This observation highlights the importance of the utilisation of other genetic targets to avoid potential false identifications and incorrect diagnostic results for *Mycoplasma* detection in clinical specimens.

M. anserisalpingitidis represents a serious threat to the geese industry worldwide (Stipkovits and Szathmary, 2012; Gyuranecz et al., 2020), therefore the detection of this agent is required for a comprehensive laboratory diagnostic analysis of goose clinical specimens. Previous attempts have been made to design primers for the detection and specific identification of this species based on the sequences of the DNA polymerase III subunit alpha (*dnaE*), elongation factor G (*fusA*), pyruvate kinase (*pyk*), and *rpoB* genes (Sprygin et al., 2012); however, those primers were not analysed for the detection sensitivity of *M. anserisalpingitidis* in avian clinical samples. The primers for the *M. anserisalpingitidis*-specific PCR assay described in this study also target the *rpoB* gene but the sensitivity of the assay was assessed to ensure its applicability for the diagnostic use.

The PCR assays developed in this study were able to confirm and/or identify these *Mycoplasma* species in new or rarely-observed waterfowl hosts. According to the available literature, *M. anserisalpingitidis* was found only in geese (Stipkovits and Szathmary, 2012); however, this species was also isolated from a domestic duck in 2012 (MYCAV 47). The previous identification was performed with the *Mycoplasma* genus-specific PCR (Lauerman et al., 1995) and this result was confirmed by the newly designed species-specific PCR assay, as well as by the DNA sequence analysis of the PCR amplicons. The partial *rpoB* gene (1997 bp long) was also sequenced for this isolate (GenBank accession number MH003302), and the nucleotide sequencing analysis demonstrated its 99% identity to the *rpoB* gene of the type strain of *M. anserisalpingitidis*. Fibrin deposition on the visceral serous membranes of the duck was also consistent with the pathological evidences observed in *M. anserisalpingitidis*-infected geese (Stipkovits et al., 1993).

M. anatis normally colonises ducks and it was rarely detected in geese (Bencina et al., 1987; Stipkovits and Szathmary, 2012). Eight *M. anatis* isolates were examined in the study out of which two isolates originated from geese (MYCAV 314 and 317). The observed pathological changes such as the fibrinous peritonitis and lymphohistiocytic infiltration in the lungs of these geese were similar to the pathological evidences reported for *M. anatis*-infected ducks (Ivanics et al., 1988). Moreover, 15% (n = 4/26) of the examined clinical specimens from geese were found to be positive for *M. anatis* using the designed species-specific PCR assays. The DNA sequence analysis of the *dnaX* amplicons from the two isolates and the four clinical specimens (Table 19) revealed their 97.7–97.9% sequence identity to the same gene of

M. anatis type strain. This high level of nucleotide identity is sufficient for the identification to the species level and demonstrates the adequate detection specificity of the *M. anatis*-based PCR assay. In addition, the partial *rpoB* gene (1994 bp long) was also sequenced for these two *M. anatis* isolates originated from geese (MYCAV 314 and 317; GenBank accession numbers MH003311 and MH003313, respectively) and the sequencing result demonstrated their 99% identity to the *rpoB* gene of the type strain of *M. anatis*. Thus, the species identity of *M. anserisalpingitidis* isolate from a duck and *M. anatis* isolates from geese was clearly demonstrated in both cases.

The developed assays discriminated the targeted waterfowl pathogen *Mycoplasma* species in the examined samples, including mixed infections. The PCR assays showed high sensitivity and specificity, enabling rapid, precise and reliable identification of these mycoplasmas, and therefore proved to be a suitable and cost-effective method in routine veterinary laboratory diagnostics. The obtained results provide valuable support for the selection and the use of adequate control and/or feasible treatment of affected birds. In addition, these assays can be run simultaneously using the same thermal cycling PCR conditions on conventional PCR equipment.

At the time of the study, as the majority of the samples originated from Hungary, further examinations on a wider selection of samples were advised to enable the determination of the robustness of the assays. Since then, the assays were used on samples from Poland, Ukraine, Vietnam (samples included in the *M. anserisalpingitidis* MLST study), and China (Gyuranecz et al. 2020), and the PCR results along with the cultured field isolates still support the reliability of the species-specific PCRs.

6.4. MLST study on *M. anserisalpingitidis*

M. anserisalpingitidis is a pathogen of geese, frequently detected in Central and Eastern European countries (Stipkovits et al., 1986; Sprygin et al., 2012); however, the occurrence of this species is not restricted to this continent (Gyuranecz et al., 2020). Information about the organ/tissue preference of *M. anserisalpingitidis* and its spatial and temporal diversity is limited, and tools for the better understanding of the phylogeny or epidemiology of this pathogen are lacking. Molecular typing techniques such as pulsed-field gel electrophoresis, restriction fragment length polymorphism, randomly amplified polymorphic DNA (Foxman et al., 2005) have become important tools to study the genetic diversity of bacterial species. MLST can provide a valuable insight into the phylogeny and molecular epidemiology of bacterial pathogens, by analysing the genetic relationship between strains and monitoring the temporal and geographical distribution of bacterial spread, identifying infection and transmission routes. Over the last few years, MLST analyses were successfully used for the

genetic characterisation of avian mycoplasmas (e.g. *M. gallisepticum* (Bekő et al., 2019), *M. iowae* (Ghanem and El-Gazzar, 2016), *M. synoviae* (Dijkman et al., 2016; El-Gazzar et al., 2017)), and other mycoplasmas of veterinary importance (Mayor et al., 2008; McAuliffe et al., 2011; Manso-Silván et al., 2012; Tocqueville et al., 2014). Furthermore, this method provides an opportunity to study infectious agents from clinical specimens without a preceding cultivation. Moreover, MLST assays show high reproducibility, and the sequence data can be shared and compared between different laboratories (Belén et al., 2009). Although, Sprygin et al. (2012) published species-specific PCR assays for the detection and genetic identification of *M. anserisalpingitidis*, these assays were not used for strain differentiation and sequence typing.

The MLST scheme described in this study was developed based on the entire genome sequences of 82 *M. anserisalpingitidis* strains and field isolates. *M. anatis* was used as outgroup because of the high similarity of the 16S rRNA genes (Volokhov et al., 2020) and the observed genome similarity of the *M. anserisalpingitidis* and *M. anatis* type strains (section 5.2). The observation, that some *M. anserisalpingitidis* field isolates' *plsY* locus is more similar to the *plsY* locus of *M. anatis* than of the other *M. anserisalpingitidis* isolates or strains is also in accordance with the theory of the common ancestor (Volokhov et al., 2020). Variation in expression and structure of surface lipoproteins (*plsY* encodes the glycerol-3-phosphate acyltransferase, a protein which is involved in the phospholipid metabolism) or horizontal gene transfer between species sharing the same ecological niche contribute to the emergence of new mycoplasma variants (Citti and Blanchard, 2013). The role of the *plsY* gene product in the pathogenesis of mycoplasmas has not been studied yet; nevertheless, an orthologue of this enzyme proved to be required for host penetration in a pathogenic fungus (Gao et al., 2013).

Although species specificity was one of the main criteria of the *M. anserisalpingitidis* MLST development, the role of the *plsY* gene in revealing clade A (the clade most closely related to *M. anatis* isolates) proved to be more important than the primers' cross-reaction between *M. anserisalpingitidis* and *M. anatis*, hence the *plsY* locus-specific primer pair was selected among the final primers. In case of direct genotyping from clinical specimens, in order to rule out the presence of *M. anatis* in the sample firstly we suggest to apply the *M. anserisalpingitidis*- and *M. anatis*-specific PCR assays (section 5.3). Further *Mycoplasma*-specific PCR assays are not necessary before the MLST assay as none of the MLST primers cross-reacted with DNA of other *Mycoplasma* species reported in waterfowl (Roberts, 1964; Stipkovits et al., 1975; Stipkovits et al., 1984b; Bencina et al., 1988; Bradbury et al., 1988).

The novel MLST scheme discriminated the examined *M. anserisalpingitidis* samples with high Simpson's index of diversity and distinguished 76 STs (Table 21). The detected high variability was a result of mainly silent mutations in the loci (239/313 synonymous SNPs in the concatenated sequences, Table 22) and the variability was confirmed by the previous cgMLST

scheme also (Kovács et al., 2020). Similarly to previous findings, i.e., pigs can be infected with multiple strains of *M. hyopneumoniae* (Vranckx et al., 2011, 2012), in the present study even the isolates collected from the same animal or flock had different STs, which showed a closer relationship based on the cgMLST scheme (Kovács et al., 2020). The high number of *M. anserisalpingitidis* variants is in accordance with the observation, that mycoplasmas are some of the fastest evolving organisms (Woese et al., 1980; Delaney et al., 2012). Nevertheless, despite the high number of STs, samples originating from the same geographical locations (e.g. isolates from Poland, or Hungarian farms not part of the examined integration) showed close genetic relationship and clustered together in the phylogenetic tree (Figure 9). The many STs observed among the isolates of the Hungarian livestock integration and their separation into different (sub)clades could be explained by the horizontal transmission (e.g. frequent animal transport or personal movement between farms) of this infectious agent.

Subclade 5C comprised the Chinese field isolates together with the sample from Vietnam, and interestingly, a few Hungarian samples, and showed the highest genetic distance within clade C. The host animal of the Vietnamese isolate was a domestic goose as in the case of the European samples; however, the Chinese isolates originated from swan geese. Although the geographical distance is considerable and the isolates originated from different animal hosts, the high similarity between the Chinese and the Hungarian isolates was also confirmed based on genetic characterization of the draft whole genomes (Gyuranecz et al., 2020). Nevertheless, the examination of a larger number of Asian samples could provide further, valuable information on the genetic background of the observed similar STs. Given the different host species, industrial geese transport probably does not explain the genetic relationship between the European and Asian isolates. As *M. anatis* and *M. cloacale* were described in wild ducks and other types of birds (Bradbury et al., 1987; Poveda et al., 1990; Goldberg et al., 1995; Samuel et al., 1995), it could not be precluded that *M. anserisalpingitidis* may spread by animal migration as well.

The *M. anserisalpingitidis* samples presented in the study were collected mainly in Hungary, as this species is frequently monitored in the country. The low number of samples from other countries probably does not mean, that this *Mycoplasma* species occurs only sporadically, rather this particular pathogen has small awareness worldwide. Further examinations based on a larger number of samples originating from several countries will be required to determine the prevalence and genetic variability of *M. anserisalpingitidis*. As *Mycoplasma* infections of waterfowl are not always associated with clinical signs (Hinz et al., 1994), but flared up mycoplasmosis could occur due to stress factors (Stipkovits et al., 1986; Stipkovits and Kempf, 1996), sampling for the detection of *M. anserisalpingitidis* is advised even from healthy birds.

The novel MLST scheme was found to be an adequate method to differentiate *M. anserisalpingitidis* samples. The developed PCR assays represent suitable and cost-effective method for routine veterinary laboratory diagnostics, and could omit the fastidious isolation process required for the cgMLST analysis. The newly developed method can be a useful genotyping tool for phylogenetic studies and future epidemiological investigations, and data generated by this typing method is directly comparable between laboratories over a web-accessible database (https://pubmlst.org/manserisalpingitidis/).

7. Overview of the new scientific results

Ad 1. Detailed antibiotic susceptibility profiles of *M. anserisalpingitidis* strains/field isolates were defined for the first time. Based on our *in vitro* examinations of thirteen antibiotics and a combination, tiamulin and valnemulin from the pleuromutilins family and the macrolide type tylvalosin proved to be the most effective drugs for the therapy of *M. anserisalpingitidis* infections in Hungary. However, isolates with elevated MIC values were detected, highlighting the importance of the susceptibility testing before treatment.

Ad 2. The type strain of *M. anserisalpingitidis* (ATCC BAA-2147) and two field isolates (MYCAV 93 and MYCAV 177), and the type strains of *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234) and *M. cloacale* (NCTC 10199) were *de novo* sequenced. The records of the complete genomes were deposited in GenBank, promoting the research of waterfowl mycoplasmosis as these were the first available complete genomes for these species.

Ad 3. Species-specific PCR assays were designed for the most common waterfowl *Mycoplasma* species. These assays were the first published for *M. anatis*, *M. anseris* and *M. cloacale*, and the first established concerning the specificity and sensitivity for *M. anserisalpingitidis*. The tests promote the rapid, single and reliable identification of the species even from DNA samples extracted from clinical specimens without the need of prior *Mycoplasma* culture. With the help of the *M. anserisalpingitidis*-specific assay, this species was detected from a duck, a previously unpublished host animal.

Ad 4. We reported seventy-nine *M. anserisalpingitidis* draft assembly from the whole genome sequencing datasets, and the previously published three complete genomes were also included in a phylogenetic study. After comprehensive *in silico* and *in vitro* examination of housekeeping genes, an MLST assay was firstly designed for *M. anserisalpingitidis*. The five loci based assay divided altogether 89 *M. anserisalpingitidis* samples into 76 STs, showing wide diversity for this species. A web-accessible phylogenetic database was established for this *Mycoplasma* species by our research group. The developed MLST scheme constitutes a universal tool for global, long-term screening of dissemination for *M. anserisalpingitidis*, promoting the phylogenetic and epidemiological investigation of this species.

8. References

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