Veterinary Microbiology 254 (2021) 108972

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Multilocus sequence typing of the goose pathogen *Mycoplasma anserisalpingitidis*

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ARTICLE INFO

Keywords:
Differentiation
Geese
MLST
Mycoplasma anserisalpingitidis

ABSTRACT

Mycoplasma anserisalpingitidis infection is associated with the inflammation of the genital tract and cloaca, embryo lethality, and decreased egg production in geese, leading to serious economic losses. *M. anserisalpingitidis* has been detected mainly in Central and Eastern Europe, especially in Hungary, but the pathogen was identified recently in China, predicting it's worldwide occurrence. In this study, a novel multilocus sequence typing (MLST) scheme was developed to analyse phylogenetic relationships between *M. anserisalpingitidis* field isolates and clinical specimens originating from different geographical locations.

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 with a 0.994 Simpson's index of diversity. The samples were originated from Hungary, Poland, Ukraine, China, and Vietnam. Phylogenetic analysis revealed the existence of three distinct clades (A–C) and six subclades within clade C. Generally, samples originating from the same geographical locations or livestock integration clustered together. Isolates in clade A showed the closest relationships to the *M. anatis* outgroup due to sequence similarity of the *plsY* locus. The highest genetic distance was observed in 5C among the subclades of clade C, containing the Asian and some Hungarian field isolates.

The developed MLST assay revealed high diversity of the investigated *M. anserisalpingitidis* samples. The method proved to be a valuable and cost-effective tool for sequence typing of this waterfowl *Mycoplasma* species, enabling the better understanding of its phylogeny and providing a robust assay for future molecular epidemiological investigations.

1. Introduction

Mycoplasma anserisalpingitidis (also known as *M.* sp. 1220) is a goose bacterial pathogen (Volokhov et al., 2020) and infection may lead to enormous economic losses in the goose industry (Stipkovits and Szathmary, 2012). It can be part of the normal microflora of the animals which do not show any clinical signs of mycoplasma infection; however, inadequate housing, crowding, sexual activity of the animals or intensive egg production can lead to the manifestation of mycoplasmosis (Stipkovits et al., 1986; Hinz et al., 1994; Stipkovits and Kempf, 1996). The main symptoms in the affected flocks are inflammation of the cloaca and genital tracts, decreased egg production and increased embryo lethality, and *M. anserisalpingitidis* has also been associated with respiratory and nervous system syndromes (Stipkovits et al., 1986, 1987,

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https://doi.org/10.1016/j.vetmic.2020.108972
Received 24 August 2020; Accepted 21 December 2020
Available online 28 December 2020

1993; Stipkovits and Kempf, 1996; Dobos-Kovács et al., 2009). The transmission of *M. anserisalpingitidis* can be horizontal including venereal or vertical (Stipkovits et al., 1984a, 1986, 1987; Dobos-Kovács et al., 2009). The pathogen has been isolated from various organs of geese, most frequently from the cloaca and the phallus of the ganders (Stipkovits et al., 1986, 1987, 1993; Hinz et al., 1994; Dobos-Kovács et al., 2009). This species can infect both domestic geese (*Anser anser*) and swan geese (*Anser cygnoides*) (Gyuranecz et al., 2020; Volokhov et al., 2020); moreover, it has been isolated also from domestic duck with *M. anserisalpingitidis* infection-like pathological conditions (Grózner et al., 2019b). *M. anserisalpingitidis* was first isolated in Hungary, Central Europe (Stipkovits et al., 1984b), and it has been reported from Germany, France, Czech Republic, Poland (Stipkovits et al., 1986), Ukraine, Russian Federation (Stipkovits et al., 1986; Sprygin et al., 2012) and China (Gyuranecz et al., 2020).

Information about the organ/tissue proclivity 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 (PFGE), restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) (Foxman et al., 2005) have become important tools to study the genetic diversity of bacterial species. Multilocus sequence typing (MLST) can provide 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 cultivation. Moreover, MLST assays show high reproducibility, and the sequence data can be shared and compared between different laboratories (Belén et al., 2009). Species-specific PCR assays were described for the detection and genetic identification of *M. anserisalpingitidis* targeting the *dnaE*, *fusA*, *pyk*, and *rpoB* genes; however, these assays were not used for strain differentiation and sequence typing (Sprygin et al., 2012). Recently, a core genome MLST (cgMLST) scheme was published for *M. anserisalpingitidis* by our research group; however, that method requires pure bacterial strains, whole-genome sequencing and high-quality input data (Kovács et al., 2020). The aim of the present study was to genetically characterize *M. anserisalpingitidis* samples with a novel MLST scheme in order to better understand the phylogeny of this *Mycoplasma* species.

2. Materials and methods

2.1. Bacterial isolates and specimens

Eighty-two *M. anserisalpingitidis* isolates, including the ATCC BAA-2147 type strain and two previously published field isolates (Stipkovits et al., 1984b; Grózner et al., 2019a; Volokhov et al., 2020) 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 (n = 40), phallus and phallus lymph (n = 30), trachea (n = 5), follicule (n = 4), semen (n = 2) and lung and air sac (n = 1) of the animals. During sample collection, flocks of a Hungarian livestock integration were sampled frequently between 2011–2018, and 36 isolates from this integration were included in this study. The distance between the sampled farms of the integration ranged from 12 to 230 km. Field isolates originating from the same flock or same animal's different organs were also included in the study (Supplementary Table 1). *M. anatis* is genetically closely related to *M. anserisalpingitidis* (Volokhov et al., 2020), thus six *M. anatis* field isolates recovered from domestic geese and ducks between 2011–2014 from Hungary were analysed with the developed assay also (Supplementary Table 1). Isolation and cell propagation were performed in Oxoid *Mycoplasma* broth medium (pH 7.8) (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 0.5 % (wt/vol) sodium pyruvate, 0.5 % (wt/vol) glucose, 0.15 % L-arginine hydrochloride and 0.05 % (wt/vol) phenol red and cultures were incubated at 37 °C.

Table 1
Amplicon sizes, analysed sequence fragment lengths and primer sequences of the target regions for the five loci studied.

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

Further seven clinical specimens (transport medium or tissue sample) without cultivation, originating from Poland (n = 6) and Ukraine (n = 1) were included in the study (Supplementary Table 1). DNA was extracted from these samples with the QIAamp DNA Mini Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturers' instructions, and *M. anserisalpingitidis* DNA was identified in the samples with the help of a species-specific PCR assay (Grózner et al., 2019b). The presence of *M. anatis* DNA was ruled out with a species-specific PCR assay (Grózner et al., 2019b).

Ethical approval and specific permission were not required for the study as all isolates and clinical specimens were collected during routine diagnostic examinations or necropsies by the authors with the consent of the owners, and sampling was not performed solely for the purpose of the study.

2.2. Whole-genome sequencing

DNA was extracted from the 82 *M. anserisalpingitidis* and six *M. anatis* isolates with the QIAamp DNA Mini Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturers' instructions. Next-generation sequencing was performed on NextSeq 500 Illumina equipment (Illumina, Inc., San Diego, CA, USA), with 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 (GenBank accession number CP042295) and M. anatis NCTC 10,156 (CP030141) using the Geneious Prime 2019.2.1 software (Kearse et al., 2012).

2.3. 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 ATCC BAA-2147 type strain were added for the analyses (Supplementary Table 2). 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 (two genomes were previously deposited under GenBank accession numbers

Table 2
The number of alleles, number of single nucleotide polymorphisms (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	Nr. of allele types	Total number of SNPs/length of the examined locus (bp)	Simpson's index of diversity
atpG	50	62/450 (13.78 %)	0.966
fusA	26	74/577 (12.82 %)	0.906
pgiB	32	33/284 (11.62 %)	0.936
plsY	41	84/325 (25.85 %)	0.957
uvrA	38	60/373 (16.09 %)	0.931
MLST scheme	76 ST[a]	313/2009 (15.58 %)	0.994

[a] Sequence type.

CP041663 and CP041664). The selected MLST loci were obtained from the NCTC 10,156 type strain (CP030141) and the whole-genome sequences of six *M. anatis* 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 described for the genetic identification of *M. anserisalpingitidis* (Sprygin et al., 2012) (Supplementary Table 2) were also investigated by *in silico* analysis using the Geneious Prime 2019.2.1 software (Kearse et al., 2012).

2.4. 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 sequence types (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.

2.5. PCR conditions, specificity and sensitivity test

All PCR reactions were carried out in 25 µl total volume, containing 5 µl 5X Green GoTaq Flexi Buffer (Promega Inc., Madison, WI, USA), 2 µl $MgCl_2$ (25 mM; Promega Inc., Madison, WI, USA), 0.5 µl dNTP (10 mM, Qiagen Inc., Hilden, Germany), 2 µl of each primer (10 pmol/µl, Supplementary Table 2), 0.25 µl GoTaq G2 Flexi DNA polymerase (5 U/µl; Promega Inc., Madison, WI, USA) and 2 µl target DNA sample. Table 1 and Supplementary Table 2 summarise primers used in this study. Thermocycling parameters were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 61 °C for 1 min and 72 °C for 1 min, completed with a final elongation step at 72 °C for 5 min. The expected molecular size of the amplicons was confirmed by electrophoresis in agarose gel stained with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics Co., Ltd, New Taipei City, Taiwan) followed by UV visualization.

Partial sequences of housekeeping genes from clinical specimens were obtained with primers listed in Table 1 by direct sequencing of the produced amplicons on 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 10,156), *M. anseris* (ATCC 49,234), *M. anserisalpingitidis* (ATCC BAA-2147), *M. cloacale* (NCTC 10,199), *M. gallinarum* (ATCC 19,708), *M. gallisepticum* (ATCC 19,610), *M. iners* (ATCC 19,705), and *M. synoviae* (NCTC 10,124).

The PCR sensitivity was evaluated with serial tenfold dilutions (10^6-10^0) of the DNA of the *M. anserisalpingitidis* ATCC BAA-2147 strain. Genomic equivalents (GE) were calculated by an online dsDNA copy number calculator (https://cels.uri.edu/gsc/cndna.html) based on the DNA concentration measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the genome size. The lowest DNA concentration yielding visible products during agarose gel electrophoresis was considered to be the detection limit for an assay.

2.6. Nucleotide sequence accession numbers

Partial sequences of the five housekeeping genes of 79 novel *M. anserisalpingitidis* field isolates 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 under accession numbers MT508595 – MT508618, and MT508625 – MT508630.

3. Results

Forty candidate MLST loci were analysed *in silico*, out of which 26 were excluded from further consideration (Supplementary Table 2). The primers described for the genetic identification of *M. anserisalpingitidis* (Sprygin et al., 2012) (Supplementary Table 2) have not been further analysed because none of them fulfilled the criteria for locus selection. 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 (Supplementary Table 2). 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 (Supplementary Table 2). 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 among the *M. anserisalpingitidis* field isolates showing closer relationship with the genetically very similar *M. anatis* samples (clade A, Fig. 1), 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 sequence types (STs) with a

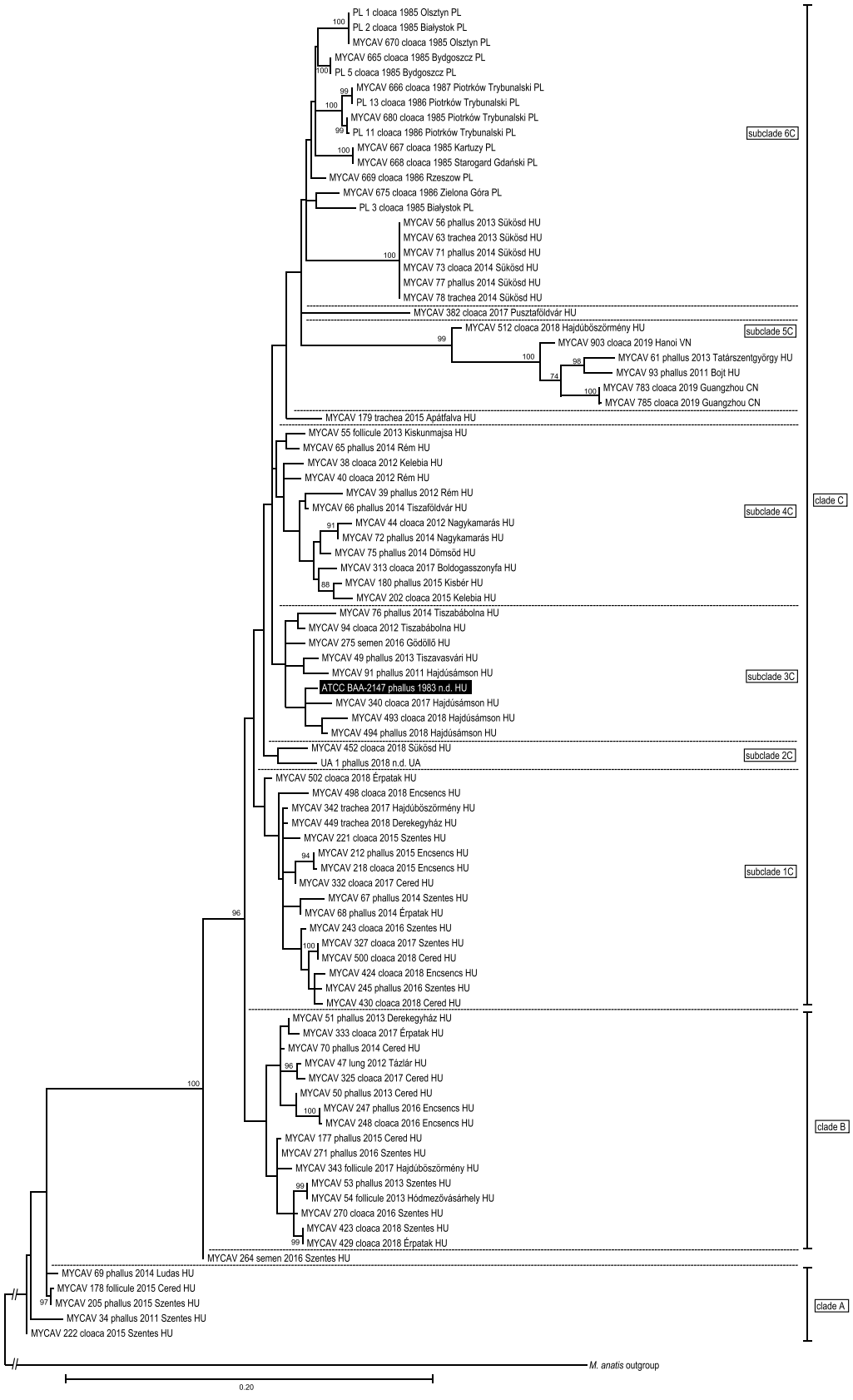

Fig. 1. Maximum Likelihood phylogenetic tree showing relationship between the investigated 89 *M. anserisalpingitidis* samples and *M. anatis* outgroup based on MLST analyses. Major clades of *M. anserisalpingitidis* are dedicated as clade A, B, and C, and subclades 1-6C. Countries are indicated as follows: 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.

0.994 Simpson's index of diversity. Allele numbers, and sequence types for all investigated *M. anserisalpingitidis* samples are described in Supplementary Table 1. A 'PubMLST' database has been set up for this MLST scheme for *M. anserisalpingitidis*. The number of unique alleles, total single nucleotide polymorphisms (SNPs), and Simpson's index of diversity for each locus is summarised in Table 2. 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 (Fig. 1).

Five Hungarian isolates were included in clade A. 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 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 (Supplementary 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 (Supplementary Table 1) had different STs.

4. Discussion

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 (Sprygin et al., 2012; Gyuranecz et al., 2020). Despite the economic losses in the goose production associated with this *Mycoplasma* species, there is still a lack of knowledge about this microorganism. Presently, there are no commercially available vaccines against *M. anserisalpingitidis*, thus the adequate housing and appropriate antibiotic treatment are promoted in the control of the diseases caused by this agent (Stipkovits and Kempf, 1996). As strains with increased resistance to antimicrobial agents have recently been described (Grózner et al., 2016), efficient genotyping tools are required for the monitoring of this bacterium. Among the bacterial typing techniques, the MLST provides high reproducibility, fast and cost-effective information about the analysed samples (Foxman et al., 2005).

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 these bacteria are genetically closely related; the nucleotide sequences of the 16S rRNA gene of the NCTC 10,156 type strain showed 99 % similarity with *M. anserisalpingitidis* ATCC-BAA 2147 type strain. These two *Mycoplasma* species may have separated from a common ancestor approximately 20–40 million years ago (Volokhov et al., 2020). 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 in accordance with the theory of a common ancestor. 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 yet been studied; 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, which could be run simultaneously (Grózner et al., 2019b). 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 before (Roberts, 1964; Stipkovits et al., 1975, 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 2). The detected high variability was a result of mainly silent mutations in the loci (239/313 synonymous SNPs in the concatenated sequences) and the variability was confirmed by the previous cgMLST scheme also (Kovács et al., 2020). Similarly to previous findings that 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 closer relationship based on the previously developed 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 from Hungarian farms not part of the examined integration) showed close genetic relationship and clustered together in the phylogenetic tree (Fig. 1). 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 about 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. *M. anserisalpingitidis*

occurrence in migratory birds have not yet been studied; however, other *Mycoplasma* species colonizing waterfowl (*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); therefore, 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*. *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), thus 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 spare the fastidious isolation process required for 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/).

Funding information

This work was supported by the Lendület program (LP2012−22) of the Hungarian Academy of Sciences, the K16 (119594), FK17 (124019) and KKP19 (129751) grants of the National Research, Development and Innovation Office, Hungary. ZK and MG were supported by the Bolyai János Research Fellowship of the Hungarian Academy of Sciences. MG was supported by the Bolyai + Fellowship (ÚNKP-19−4-ÁTE-1) of the New National Excellence Program of the Ministry of Innovation and Technology. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2020.108972.

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