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Development of molecular assays for the analysis of genetic relationships of *Mycoplasma iowae*

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ABSTRACT

Mycoplasma iowae is a worldwide spread and economically important avian pathogen that mostly infects turkeys. Currently, multi-locus sequence typing (MLST) serves as the gold standard method for strain identification in *M. iowae*. However, additional robust genotyping methods are required to effectively monitor *M. iowae* infections and conduct epidemiological investigations. The first aim of this study was to develop genotyping assays with high resolution, that specifically target *M. iowae*, namely a multiple-locus variable number of tandem-repeats analysis (MLVA) and a core genome multi-locus sequence typing (cgMLST) schema. The second aim was the determination of relationships among a diverse selection of *M. iowae* strains and clinical isolates with a previous and the newly developed assays. The MLVA was designed based on the analyses of tandem-repeat (TR) regions in the six serotype reference strains (I, J, K, N, Q and R). The cgMLST schema was developed based on the coding sequences (CDSs) common in 95% of the examined 99 isolates. The samples were submitted for a previously published MLST assay for comparison with the developed methods. Out of 94 TR regions identified, 17 alleles were selected for further evaluation by PCR. Finally, seven alleles were chosen to establish the MLVA assay. Additionally, whole genome sequence analyses identified a total of 676 CDSs shared by 95% of the isolates, all of which were included into the developed cgMLST schema. The MLVA discriminated 19 distinct genotypes (GT), while with the cgMLST assay 79 sequence types (ST) could be determined with Simpson's diversity indices of 0.810 (MLVA) and 0.989 (cgMLST). The applied assays consistently identified the same main clusters among the diverse selection of isolates, thereby demonstrating their suitability for various genetic analyses and their ability to yield congruent results.

1. Introduction

Mycoplasma iowae is an economically important pathogen, which causes reduced hatchability (ranging between 2% and 10%), late embryo mortality, leg deformities, chondrodystrophy and skeletal lesions (Wood and Wilson, 2013; Bottinelli et al., 2021). *M. iowae* infection mainly occurs in turkeys but it has been described in chickens, geese, game and exotic birds (Catania et al., 2012, 2014). In the past, six distinct serotypes of *M. iowae* (I, J, K, N, Q and R) have been distinguished from other mycoplasmas with growth inhibition,

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hemagglutination and serum plate agglutination tests (Aycardi et al., 1971). However, due to its high antigenic variability and the evoked weak humoral response, there are no available serological tests to detect *M. iowae* (Panangala et al., 1992; Kempf et al., 1994; Wood and Wilson, 2013). Control of the disease is problematic also, since vaccines are unavailable and the pathogen is naturally more resistant to antimicrobials than other avian pathogen mycoplasmas (Gautier-Bouchardon et al., 2002). Due to the huge economic losses in the past, eradication programmes were conducted; nevertheless, several outbreaks have been reported in commercial turkey stocks in the United States and Europe in recent decades (Catania et al., 2012; Nemes et al., 2019). Consequently, the epidemiological monitoring of the pathogen has a substantial role.

Molecular assays are the most efficient tools for the identification and differentiation of *M. iowae* strains, isolates and clinical samples (Ghanem and El-Gazzar, 2016). Various methods have been developed in the past to discriminate *M. iowae*, such as Restriction Fragment Length Polymorphism (RFLP) (Zhao and Yamamoto, 1989) and Random Amplified Polymorphic DNA (RAPD) (Fan et al., 1995). These methods need the isolation of the bacteria in pure culture, which can be difficult in the case of mycoplasmas; moreover, fingerprinting assays often yield low reproducibility. Therefore, alternate typing methods were required to investigate outbreaks, intraspecies heterogeneity of *M. iowae* and determine the source of infection. Currently, multi-locus sequence typing (MLST) (Ghanem and El-Gazzar, 2016) is considered the gold standard for genetic characterisation of *M. iowae*. MLST is a sequence-based method with high reproducibility and resolution. The *M. iowae* specific MLST evaluates single nucleotide polymorphisms (SNPs) in six targeted housekeeping genes (*dppC, kdpA, leuS, rpoC, ulaA, valS*). An additional advantage of MLST over fingerprinting methods is that it does not require the isolation of the bacteria. In recent years, several genotyping methods have been developed for avian mycoplasmas, including the multiple-locus variable number of tandem-repeats analysis (MLVA) and the core genome multi-locus sequence typing (cgMLST) of *M. synoviae* (Ghanem and El-Gazzar, 2018; Kreizinger et al., 2018), and cgMLST and MLST of *M. gallisepticum* (Ghanem et al., 2018; Bekő et al., 2019) and *M. anserisalpingitidis* (Kovács et al., 2020; Grózner et al., 2021).

MLVA is a powerful molecular typing method based on repetitive DNA elements in bacterial genes or in intergenic regions. It is considered a convenient, high-throughput, high-speed method for microbial strain identification (Nadon et al., 2013). A whole-genome sequencing (WGS) based typing approach, the cgMLST method allows a highly discriminatory comparison of the similarity of bacterial genomes (Deneke et al., 2021). WGS-based methods offer superior discriminatory power compared to conventional molecular typing methods, and with the declining costs of next generation sequencing, their utilisation has expanded in outbreak investigations, as well as in comparative and evolutionary genetic studies (Ghanem and El-Gazzar, 2018).

The aims of this study were to develop MLVA and cgMLST assays specific for *M. iowae* and to determine the phylogenetic relationships of 99 isolates using these genotyping assays.

2. Methods

2.1. Samples

A total of 99 clinical isolates and strains were used for the examination, which included the type strain (NCTC 10185), the reference strains of serotypes I (strain Iowa 695), R (D2497), N (PHN-D13), J (693), Q (L3–10) and K (1805) and strain PPAV. The NCTC 10185 type strain (Iowa 695) and the serotype I strain (Iowa 695) were received from different laboratories. The isolates were collected from five different host species (chickens, ducks, partridges, pheasants, and turkeys) and from 10 distinct organs or sample types (cloaca, egg, embryo, esophagus, eye, intestinal tract, meconium, oropharynx, seminal fluid, and trachea). One field isolate is from Canada from 2006, two from Croatia from 2019, one from France from 1982, 11 from Hungary (isolated between 2016 and 2019), seven from Israel (isolated between 1990 and 1996), 38 from Italy (isolated between 2010 and 2018), one from Pakistan from 1990, 23 from the United Kingdom (isolated between 1980 and 2013) and eight from the United States of America (isolated between 1990 and 1998) (Supplementary Table 1). The isolates were cultured as described before (Buni et al., 2022).

2.1.1. Whole genome sequencing and assembly

Apart from the publicly available WGS of the *M. iowae* type strain (NCTC 10185, GenBank Accession number: NZ_LR215023), a total of 98 WGSs were determined in the present study and analyzed. The 98 *M. iowae* isolates were propagated in 8 ml liquid media each, and centrifuged after color change at 9000 x g for 9 min. The sediments were submitted to DNA extraction with the QIAmp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturers' instructions. The six *M. iowae* serotype reference strains have been sequenced using Oxford Nanopore MinION Mk1C (Oxford Nanopore Technologies Ltd, Oxford, UK) instrument and Illumina NextSeq 500 platform (Illumina Inc., San Diego, CA, USA) to generate both long-reads and short-reads. The quantity of purified DNA was measured by Qubit fluorometer with Qubit dsDNA BR Assay Kit (Thermo Scientific, Waltham, MA, USA). The starting input DNA concentration was 1000–1200 ng and 1 ng (0.2 ng/µl) per sample for long-read sequencing and for short-read sequencing, respectively. Library preparation for MinION was performed by Ligation sequencing kit (SQK-LSK109) with native barcoding expansion kit (EXP-NBD104) according to the manufacturer's instructions. Sequencing was performed on a R9.4.1 SpotON Flow Cell (FLO-MIN106). For short-read sequencing, library was prepared with the Nextera XT DNA Library Preparation Kit and the Nextera XT Index Kit v2 Set A as described in details elsewhere (Bali et al., 2021). To gain 150 bp single reads, the library pool at a final concentration of 1.8 pM was loaded onto a NextSeq 500/550 High Output flow cell. The remaining strains (n = 92) were sequenced using Illumina NextSeq 500 platform with the same parameters.

The six *M. iowae* reference strains' sequences were first de novo assembled with Flye software (version 2.8.3-b1695) (Kolmogorov et al., 2019) and then corrected using short read mapping in Geneious Prime software (version 2022.2.2) (Kearse et al., 2012). Draft genomes were assembled for the rest of the strains using the short reads from the whole genome sequencing of 92 *M. iowae* strains with the SPAdes software (version 3.13.1) (Bankevich et al., 2012). To compare the quality of the results from the two types of sequencing (Oxford Nanopore and Illumina) the Phred scores and the coverage of the reads were evaluated in the case of the six strains where both methods were applied.

2.2. Development of MLVA

Tandem-repeat (TR) regions were identified in the whole genome sequences of the *M. iowae* type strain NCTC 10185 and the six reference strains (I, J, K, N, Q and R) with the help of the Tandem Repeat Finder program (Benson, 1999) and by the analysis of the locally collinear blocks (LCBs) determined with the Mauve alignment method (Darling et al., 2004). The loci for the MLVA were selected based on the period size (at least 12 and maximum 87 bp) and the absence of insertions and deletions (indels 0%). Primers flanking the TR regions were also designed based on the type strain and the serotype strains. The melting temperature of the primers and the general suitability was calculated with NetPrimer software (Premier Biosoft International, Palo Alto, CA). The specificity of the primers was checked for cross-reactivity to against other species with BLASTN (https://blast.ncbi.nlm. nih.gov/Blast.cgi). To test the suitability of the primers and the diversity of the selected TR regions, preliminary amplifications with eight strains of highly diverse origin and with the type strain were performed (Supplementary Table 1). For further analysis, eight TR regions were selected and tested on 99 *M. iowae* strains. The PCR mixtures contained 2.5 µl GoTaq Green

Buffer (Promega Corporation, Madison, WI, USA), 2.5 µl $MgCl_2$ (25 mM, Promega Corporation) 0.5 µl dNTP (10 mM, Qiagen), 1 µl of each primer (10 pmol/µl), 0.25 µl 5x GoTaq Flexi DNA polymerase (Promega Corporation), 2 µl DNA template and nuclease-free water to a final volume of 25 µl. The PCR was performed on Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories). The thermocycling parameters were 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 50 °C/54 °C for 30 s and 72 °C for 45 s. A final elongation step was performed at 72 °C for 5 min. During the primer design the aim was to adjust similar annealing temperatures. The annealing step of the PCR assays using MI608 and MI166 primers ran at 54 °C, while all the other assays ran at 50 °C. For the electrophoresis, 5 µl of the reaction mixture were used. TR sizes above 37 bp were detected in 2% agarose gel (SeaKem LE Agarose, Lonza Inc., Rockland, ME, USA), while TR sizes below 37 bp were detected in 3% agarose gel (Metaphor Agarose, Lonza Inc.). For amplicons above 250 bp, a 100 bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fischer Scientific, Waltham, MA, USA), and below 250 bp a 20 bp (O'RangeRuler 20 bp, Thermo Fischer Scientific) DNA ladder was used as molecular weight marker. The amplified PCR products were visualized with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics Inc, Torrance, CA, USA) under UV light. Stained gels were photographically documented (Kodak Inc., Rochester, NY, USA) and the band sizes were measured with the Kodak MI SE software package (Kodak Inc.). The band sizes were converted to the number of TR. The clustering analysis was performed in MEGA 11 software (Tamura et al., 2021) with Neighbor-Joining method based on pairwise distances.

2.3. Sensitivity and specificity of the assays

The sensitivity of the assays was checked with the 10-fold dilution series of the *M. iowae* type strain in the range of 10^6-10^1 template copy number/µl. The template copy number was calculated with an online tool (Staroscik, 2004) based on the DNA concentration extracted from the pure *M. iowae* culture, which was measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). The lowest copy number that could produce a PCR amplicon that could be detected on an agarose gel was considered the detection limit of the assay. The specificity of the assays was tested including the following *Mycoplasma* species: *M. anatis, M. anseris, M. anserisalpingitidis, M. cloacale, M. columbinasale, M. columborale, M. gallinaceum, M. gallinarum, M. gallisepticum, M. gallopavonis, M. iners M. meleagridis, M synoviae.* For further evaluation, the MLVA was challenged with DNA extracted from 16 clinical samples, where *M. iowae* positivity was confirmed by TaqMan qPCR assay (Raviv and Kleven, 2009) (Supplementary Table 2).

2.4. Setting up the core genome MLST (cgMLST) schema

The *M. iowae* cgMLST schema was created as described in a previous study (Kovács et al., 2020). In brief, the previously generated 98 draft genomes and the publicly available WGS of the type strain were used in the study, and the cgMLST schema was set up using the chewBBACA software (version 3.1.0) (Silva et al., 2018). Initially, a whole genome MLST (wgMLST) schema was created, which contained all the CDSs that were found in all strains. Subsequently, the cgMLST schema was derived from the wgMLST schema by applying a 95% presence threshold, including only those CDSs that were present in 95% of the strains. Finally, a phylogenetic tree was created using the Neighbor-Joining method in Grapetree software (version 1.5.0) (Zhou et al., 2018).

2.5. Comparison of the developed assays

The isolates were analyzed based on the previously published MLST (Ghanem and El-Gazzar, 2016), and for the comparison of the isolates with an even wider selection of samples a phylogenetic tree was also created with the sequences available in the pubMLST database (https://pubmlst.org/) (Supplementary Figure 1). To evaluate the discriminatory power of the developed MLVA and cgMLST systems in relation to the previously published MLST, the Comparing Partitions online tool, (http://www.comparingpartitions.info/) was employed. The discriminatory power of the three methods was determined based on the Simpson's diversity index. Furthermore, the Adjusted Rand and Adjusted Wallace coefficients were calculated to provide quantitative measures of congruence among the three assays.

3. Results

3.1. Whole genome sequencing and assembly

The WGSs of a total of 98 *M. iowae* strains were determined in this study, six strains (Iowa 695, 693, 1805, PHND-13, L3–1 and D2497) on both Illumina and Oxford Nanopore platforms, 92 isolates only with Illumina sequencing. The Oxford Nanopore sequencing yielded an average of 135 742 sequence reads (minimum: 61 808, maximum: 338 422). The average Phred score of the six Oxford Nanopore sequencing was 19.75 (minimum: 19.2, maximum: 20.3), which corresponds to approximately 99.00% accuracy (approximately a single mistake can be expected in every 100 nucleotides). The coverage of the Oxford Nanopore sequencing was on average: 283.8x (minimum: 136.6x maximum: 612.1x). The average Phred score of the Illumina sequences for the six strains was 34.0 (minimum: 33.2 maximum: 33.5), which corresponds to approximately 99.95% accuracy. The coverage of the Illumina sequencing was on average: 283.8x (minimum: 136.6x maximum: 612.1x). The overall quality and length of the sequences from the Oxford Nanopore platform (minimum read length: 93 bp, maximum read length: 128 181 bp) were suitable to assemble a complete and circular genome in each case. However, the complete genomes would still contain a high amount of sequencing error (with a complete genome of approximately 1 250 000 bp, the 99.0% accuracy results in about 12 500 errors) using only the Oxford Nanopore technique, which is especially undesirable during the development of genotyping assays. Therefore, Illumina sequencing was required in order to increase the precision of the determined sequences. The whole genome assembly and short read mapping correction of the six strains resulted in 1 271 687 bp long sequences on average (minimum: 1 233 833, maximum: 1 333 558), with an average GC content of 24.6%.

On average the Illumina sequencing resulted in 4 630 187 short reads (minimum: 1 475 807 short reads, maximum: 9 452 424 short reads), between 35 and 151 bp. The average number of contigs per strains from the SPAdes assembly was 469. The average GC percentage was 25.3%. The average Phred score of the Illumina sequences was 33.8 (minimum: 31.8 maximum: 35.7), which corresponds to approximately 99.96% accuracy. The coverage of the Illumina sequencing was on average: 396x (minimum: 126x maximum: 842x). The sequences have been deposited in the GenBank database (Bioproject number: PRJNA975348 and GenBank Accession No.: CP129199, CP129196, CP129194, CP129197, CP129195, CP129198).

3.2. Development of MLVA

A total of 82 TRs were initially identified in the WGS of the *M. iowae* type strain. This number was reduced to 32 after considering the length and the presence of indels in the repeat regions. An additional 15 TR regions were omitted because the flanking regions were not suitable for primer design. After preliminary amplification of the type strain and eight highly diverse strains from Canada, Croatia, Israel, Italy, Hungary, Pakistan, United Kingdom and United States of America from cloaca and trachea samples (Supplementary Table 1) four markers turned out to be monomorphic, and multiple bands were detected in six cases. Ultimately, seven alleles were selected (Table 1, Fig. 1). The detection limit was 10^3 template copy numbers/reaction in all assays, and there were no cross reactions with other avian mycoplasmas. Clinical samples showed diverse DNA load (range of Ct values: 25–37), but their MLVA

Table 1
Characteristics of the tandem-repeats (TR) and the primer sequences of the developed multiple-locus variable number of tandem-repeats analysis. Abbreviations: bp basepairs; CI confidence interval.

TR name	Primer name	Primer sequence (5′−3′)	Product length	Repeat size	Consensus TR sequence	**Simpson's Index of Diversity (CI 95%)**
MI246	MI246-TR18-F	CAGATCATCTAAATGTTCTA	112 bp	28 bp	TTATTAATTATTTGTATATTGCACTATT	0.406 (0.321–0.492)
	MI246-TR18-R	AAACATTTTAATCTTGTAATC				
MI608	MI608-TR22-F	ACTAATCCAAATACACAAAA	280 bp	37 bp	TAAGTAAAGAAGAAGCTTATAATTACTTTACATATGT	0.263 (0.159–0.366)
	MI608-TR22-R	GTGCTATCATTTTTATCTGT				
MI818	MI818-TR40-F	CTTTAACTTGTTCGTGTTC	393 bp	18 bp	TTACTTGGTTCATTTGGA	0.271 (0.160–0.382)
	MI818-TR40-R	TACTAGATCTTAATATCGGTT				
MI994	MI994-TR42-F	GCTCCTTTATTACTGTCACT	419 bp	12 bp	GGTGATGGAATA	0.020 (1.000–0.060)
	MI994-TR42-R	TTTGGTTTTACTTGTTCTTG				
MI204	MI204-TR45-F	CATTTTCTTGATTTTGATTAC	296 bp	87 bp	ACATCATCAAAATTTTCAAAATTAGATTTTGTATCATTTAAATTAGCAACAACCTCTTGATTATATCCACCAAATTTACTATCAAGC	0.020 (1.000–0.060)
	MI204-TR45-R	TATGGTTCCTTTTGATTTAG				
MI123	MI123-TR46-F	AGAAAATAATAATACTGAAAA	158 bp	15 bp	AACATTAAAGATAAT	0.505 (0.503–0.507)
	MI123-TR46-R	AATCATCAATGGAAACAAA				
MI166	MI166-TR47-F	TTTCTAATGATGTTGCTTGC	359 bp	33 bp	AATCATTTAAATCTGAGATAAAATCAGATATGG	0.740 (0.709–0.770)
	MI166-TR47-R	TTCTACCATATCTGATTTGATTT				

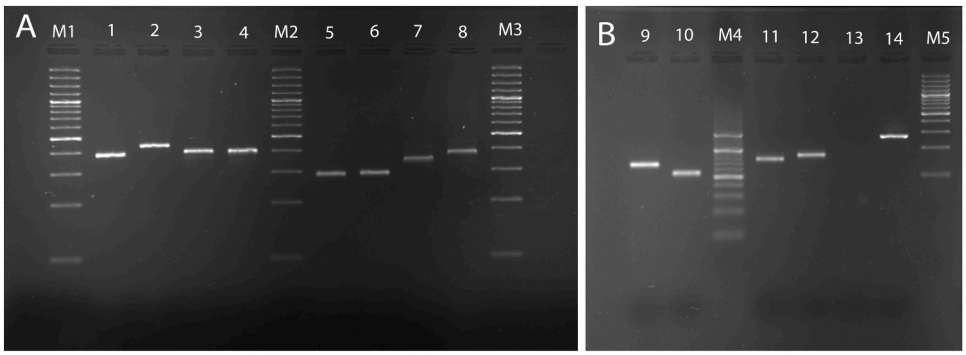

Fig. 1. Example gel for multiple-locus variable number of tandem-repeats analysis (MLVA). A: The PCR products of *Mycoplasma iowae* strains BO-B (Columns 1, 3, 5, 7) and NL-12 (Columns 2, 4, 6, 8) amplified with the primers specific for MI818 (Columns 1–2), MI994 (Columns 3–4), MI204 (Columns 5–6) and MI166 (Columns 7–8) TRs in 3% agarose gel (Metaphor Agarose, Lonza Inc.). The 100 bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fischer Scientific) is used as molecular weight marker in Columns M1, M2 and M3.B: The PCR products of strains BO-B (Columns 9, 11, 13) and NL-12 (Columns 10, 12, 14) amplified with the primers specific for MI246 (Columns 9–10), MI123 (Columns 11–12) and MI608 (Columns 13–14) TRs in 3% agarose gel (Metaphor Agarose, Lonza Inc.). The 20 bp DNA ladder (O'RangeRuler 20 bp, Thermo Fischer Scientific) is used as molecular weight marker in Column M4, and the 100 bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fischer Scientific) in Column M5.

profiles could be clearly determined. No sign of cross reaction or multiple bands was detected, except in the case of allele M166, where additional amplicons were observed at 174 bp and above 1000 bp in 10 clinical samples (Supplementary Table 2). The length of the flanking region in this allele is 95 bp and the size of the tandem repeat is 33 bp (Table 1); therefore, the additional amplicons we observed were considered the products of non-specific primer binding.

3.3. Setting up the core genome MLST (cgMLST) schema

The chewBBACA software identified 1615 CDSs for the *M. iowae* wgMLST schema. The cgMLST schema consisted of 676 CDSs, representing 67.8% of the full CDS set of the type strain NCTC 10185, with a 95% presence threshold. The most conservative alleles, present in all strains, exhibited a single variant (NCTC 10185-protein47, NCTC 10185-protein366, NCTC 10185-protein594, NCTC 10185-protein676, NCTC 10185-protein811), while the most variable allele showed 34 different genotypes (NCTC 10185-protein14). However, this highly variable allele was present in only 95.96% of the strains (Supplementary Table 3).

3.4. Comparison of the methods

3.4.1. Overall and statistical comparison of the methods

The newly developed MLVA assay successfully discriminated 19 distinct genotypes (GT), while the previously published MLST (Ghanem and El-Gazzar, 2016) and the developed cgMLST assays identified 24 and 79 sequence types (ST), respectively. Simpson's diversity indices were calculated to be 0.810 for the MLVA, 0.864 for the MLST and 0.989 for the cgMLST (Table 2). The Adjusted Rand coefficients were 0.687 for the MLVA-MLST, 0.057 for the MLVA-cgMLST and 0.114 for the MLST-cgMLST. The Adjusted Rand and Adjusted Wallace coefficients (Table 3) indicated high congruency and interchangeability between the newly developed MLVA and MLST, while cgMLST demonstrated poor agreement with MLVA and MLST. The topology of the dendrograms was highly similar among all systems. Four main clusters consisting of the same strains could be distinguished on the dendrograms assessed from all methods (Clusters A-D, Figs. 2 and 3).

Table 2
Simpson's index of diversity with 95% confidence interval (CI) and 95% non-approximated confidence interval (CINA) of the three molecular typing methods based on the analyses of 99 *M. iowae* strains.

Name	# partitions	Simpson's ID	CI (95%)	CINA (95%)
cgMLST	79	0.989	0.981–0.998	0.980–0.999
MLST	24	0.864	0.813–0.915	0.812–0.916
MLVA	19	0.810	0.761–0.859	0.759–0.860

Table 3
Adjusted Wallace coefficients with a 95% confidence interval of the three molecular typing methods based on the analyses of 99 *M. iowae* strains.

	MLVA	MLST	cgMLST
MLVA		0.574 (0.444–0.705)	0.030 (0.000–0.065)
MLST	0.856 (0.737–0.975)		0.061 (0.005–0.117)
cgMLST	0.644 (0.355–0.933)	0.889 (0.745–1.000)	

3.4.2. Analysis with respect to different geographical locations

Clustering of the strains was primarily observed based on their country of origin, with potential correlations to the year of the isolation and host species. Clinical isolates from the three continental European countries (Croatia, Hungary, Italy) showed close relationship with all three typing methods. In cluster A, the Hungarian and the Croatian strains from an epidemic in 2018–2019 formed a distinct subclade on the cgMLST tree, while they shared the same ST/GT with the remaining isolates in this cluster on the MLST and MLVA trees (Figs. 2 and 3).

Clusters A and C contained all the Italian strains with three exceptions: IZSVE/2555–4 F (isolated in Italy from incubated turkey eggs in 2011) and two isolates from the same year and host: IZSVE/1761–4 F and IZSVE/4082–4 F (Italy, turkey, 2014) belonged to cluster B on all three dendrograms. These three strains were closely related to the Italian turkey strains in cluster A (ST22/GT19), differing on three alleles with MLVA and altogether in six SNPs on three MLST loci. The Italian isolates from game birds were all included in Cluster C. Besides strains from partridges and pheasants, only a single Italian turkey isolate (IZSVE/365–2D) was grouped in Cluster C, originating from a farm which had no contact with game birds based on the available background information.

Strains from the United Kingdom and North America were grouped into clusters B and D, with four exceptions: B100/12 (UK, turkey, 2012) and B95/09 (UK, turkey, 2009) which were positioned among the Italian strains in cluster A (ST22 and GT19) and isolates B8/07 (UK, turkey, 2007) and B23/13 (UK, turkey, 2013) which were located on the same main branch with the type strain. On the MLVA tree, the latter two isolates formed an independent GT group (GT6) with B2/90 (Pakistan, chicken, 1990), while on the MLST and cgMLST trees, they were placed

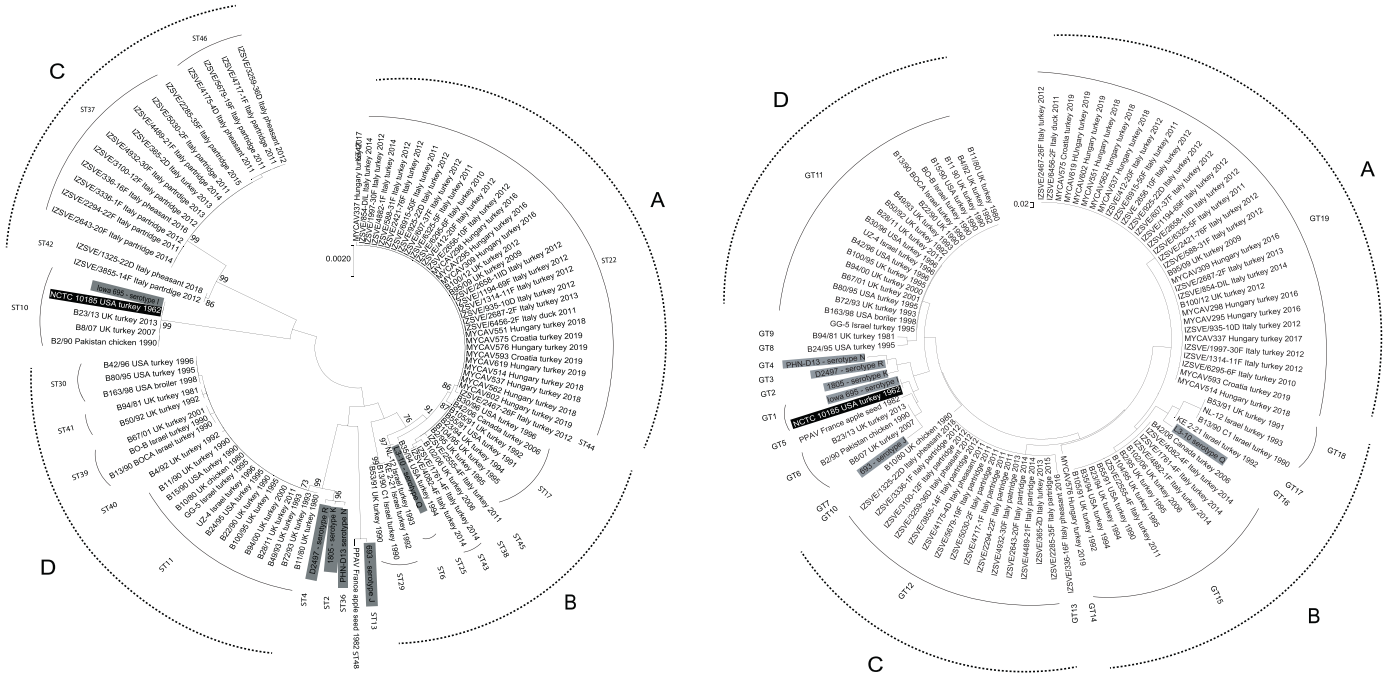

Fig. 2. Phylogenetic analysis of 99 *M. iowae* strains using multi-locus sequence typing (MLST) and multiple-locus variable number of tandem-repeats analysis (MLVA) methods.
The alignment of the concatenated sequences from the MLST assay (Ghanem and El-Gazzar, 2016) was analysed in MEGA 11 software (Tamura et al., 2021) using Maximum Likelihood method with 100 bootstrap replications and the Hasegawa-Kishino-Yano substitution model. Bootstrap values greater than 70 are shown on the dendrogram. The analysis of the MLVA profiles were performed with Neighbor-Joining method based on pairwise distances in the MEGA 11 software (Tamura et al., 2021). The type strain NCTC 10185 (black) and the serotype strains Iowa 695, 693, 1805, PHND-13, L3–10 and D2497 (grey) are highlighted on the dendrograms.

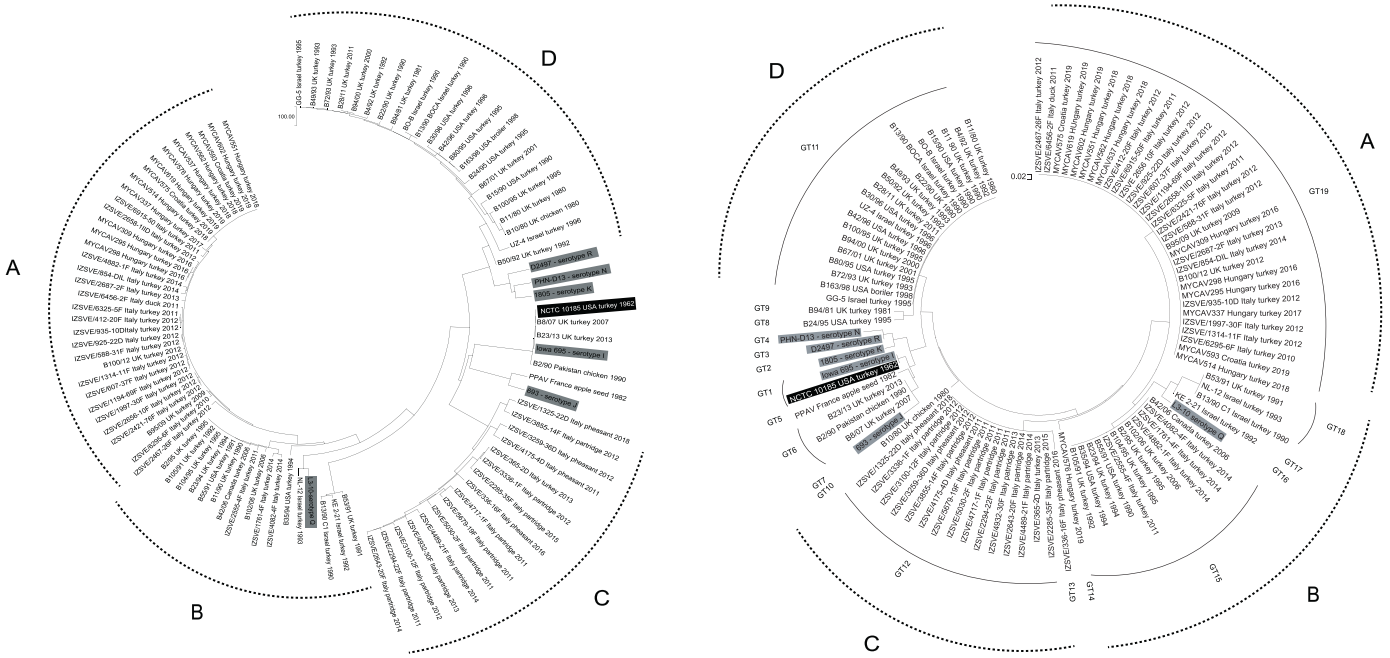

Fig. 3. Phylogenetic analysis of 99 *M. iowae* strains using multiple-locus variable number of tandem-repeats analysis (MLVA) and core-genome multi-locus sequence typing (cgMLST) methods. The cgMLST phylogenetic tree was created using the Neighbor-Joining method in Grapetree software (version 1.5.0) (Zhou et al., 2018). The analysis of the MLVA profiles was performed using Neighbor-Joining method based on pairwise distances in the MEGA 11 software (Tamura et al., 2021). The type strain NCTC 10185 (black) and the serotype strains Iowa 695, 693, 1805, PHND-13, L3–1 and D2497 (grey) are highlighted on the dendrograms.

on the same branch as the type strain.

3.4.3. *Analyses with respect to reference strains and clinical samples*

On the phylogenetic trees created by all three methods, it was observed that D2497 (serotype R), 1805 (serotype K) and PHN-D13 (serotype N) were located close to each other, differing from each other by only two MLST loci (altogether three SNPs) and three MLVA alleles. L3–10 (serotype Q) shared a common ST with NL-12 strain

(Israel, turkey, 1993) on the MLST tree, while with the MLVA and cgMLST methods, it formed an independent branch. Serotype Q strain L3–10 exhibited the greatest difference from the other serotypes on all trees; moreover, this was the only serotype that fit in a main cluster (cluster B) (Figs. 2 and 3). It was closely related on MLST and cgMLST trees with the PPAV (France, apple seed, 1982) strain. Although the reference strain of Serotype I, Iowa 695 is the same as the one deposited in the National Collection of Type Cultures (NCTC, United Kingdom), our copies differ with cgMLST, probably due to the high number of passages of this frequently used strain.

Among the 15 clinical samples from 2018, which originated from the same outbreak as the Hungarian strains from 2018 (MYCAV514, MYCAV537, MYCAV551, MYCAV562), all shared the same MLVA profile (Supplementary Table 2). The clinical sample 23107/K2/D from 2023 differed from 2018 samples by only one allele, thus forming a new GT.

4. Discussion

Mycoplasma iowae is one of the economically most important avian mycoplasmas in turkeys. The molecular typing methods play crucial role in the control of the infection and establish better understanding of the epidemiology of the pathogens. The present study provides the first, high resolution MLVA and cgMLST methods for the genetic characterization of *M. iowae* which represent new possibilities to execute epidemiological investigations and help to identify the source of the outbreaks. Besides, they allowed the detailed phylogenetic analysis of a temporally and geographically diverse selection of *M. iowae* isolates.

The developed MLVA method represents a rapid, repeatable, easily accessible, cost-effective approach, suitable for limited laboratory conditions as well. The MLVA method does not require isolation of the microorganism, the examination could be carried out directly from the DNA. This method identifies the copy number of repeated DNA sequences with high mutation rate that are dispersed throughout the bacterial genome, therefore, it could detect short term evolutionary relations among strains (Nadon et al., 2013). In the present study, the VNTR regions of the examined 99 *M. iowae* isolates exhibited limited variability, as the established assay could identify only 19 distinct GTs. Despite the relatively low Simpson's index of the MLVA (which corresponded with the resolution of the MLST assay), the dendrogram based on this method resulted similar topology and clustering of the isolates with the ones gained by MLST and cgMLST methods (Figs. 2 and 3). Notably, MLVA was the only system capable of differentiating the type strain from the B23/13, B8/07 and B2/90 field strains, hence, the developed MLVA is assumed to be a suitable method for fine-scale typing of *M. iowae*.

The Adjusted Rand coefficient provides a measure of the overall agreement of two typing methods, while the Adjusted Wallace coefficient provides information about the directional agreement between typing methods and gives confidence intervals (CI) (Severiano et al., 2011). Based on the congruencies between the two systems (Table 3), the newly developed MLVA assay is an appropriate alternative for the existing MLST. The validation of the MLVA confirmed its suitability for the direct examination of clinical samples, as non-specific amplicons could be detected only in the most variable allele (M166, based on Simpson's diversity indices, Table 1), which could be clearly distinguished from the specific bands.

The cgMLST method offers higher discriminatory power compared to other conventional typing methods and allows for precise analyses of the epidemiological correlations (Ghanem and El-Gazzar, 2018). The cgMLST assay developed in this study proved to be a robust and high-resolution method for genotyping *M. iowae*. Among the three genotyping methods, cgMLST exhibited the highest resolution and robustness in the analyses of the 99 *M. iowae* strains (Table 2, Fig. 3). The most closely related Italian and Hungarian strains in cluster A were effectively distinguished exclusively by the cgMLST. These results underscore the efficiency of cgMLST as a typing method for *M. iowae* strains. However, it requires high-quality whole genome sequences from pure cultures, therefore the expenses and requirements are higher than in the case of the MLVA and MLST.

The developed typing systems identified the same four main clusters among the examined 99 isolates, differentiated the serotype reference strains and the type strain from the majority of the examined strains and correlations were revealed between the genetic properties of the strains and the host or the country of origin (Figs. 2 and 3). The strains obtained from game birds (reared for hunting) formed a single main cluster (cluster C) on the phylogenetic trees, suggesting the circulation of a distinct lineage among this particular industrial poultry species. Our data showed that *M. iowae* GT12 (within cluster C) was detected in Italy during a longer time period (from 2011 to 2018), but the epidemic wave observed in the Italian turkey sector between 2012 and 2014 (Catania et al., 2012; Bottinelli et al., 2021) was related to different genotypes. Taking this into account and considering also that turkey, broiler, layer, pheasant, partridge and grey partridge farms coexist in the Italian densely populated poultry area (DPPA), it is plausible that host-specific *M. iowae* genotypes may exist. Alternatively, it is possible that horizontal spread of *M. iowae* between the two poultry sectors (game bird and turkey) is not as effective as it was observed in the case of *M. gallisepticum*. Epidemiological studies conducted on *M. gallisepticum* (Bekő et al., 2019; Matucci et al., 2020) revealed that the same ST was present in different poultry species of the same DPPA, meaning that this pathogen easily spreads horizontally and host-specific lineages cannot be distinguished.

The Hungarian *M. iowae* strains originating from an outbreak in Western-Hungary in 2018 and 2019 (Nemes et al., 2019), along with the Croatian strains from 2019, shared the same MLVA, MLST and cgMLST profiles. These strains formed a distinct sub-clade within cluster A in the cgMLST analysis. Similarly, the Italian turkey strains displayed low variability in the MLVA and the MLST assays, with most of them classified within cluster A, although a few closely related exceptions were observed. The cgMLST data within cluster A show that a specific clade well adapted to the turkey sector was present in some EU countries in recent years. This can be the consequence of the lack of European surveillance program for *M. iowae* infection, unlike for *M. gallisepticum* and *M. meleagridis* infections [by 2021 Regulation (EU) 2016/429 and Commission Delegated Regulation (EU) 2019/2035 whereas in the past Directive 2009/158/EC], as the recovery of *M. iowae* derives mostly from clinical cases (Bottinelli et al., 2021) and the infection may be underestimated. Alternatively, the observed low diversity is connected with the vertical transmission of *M. iowae* and the organization of the industrial turkey husbandry, with farms importing poults from the same couple of hatcheries. Certainly, additional studies will be needed to clarify these intriguing aspects, even though it is important to remember that these new typing systems can give us answer but also further questions. Out of the three turkey isolates (IZSVE/2555–4 F, IZSVE/1761–4 F and IZSVE/4082–4 F) which were grouped in cluster B one was isolated from incubated turkey eggs which did not originate from Italy. The other two isolates lack the additional metadata which would allow us to understand better their "exceptional" genotypes. Nevertheless, there is no doubt that these isolates appear to be slightly different from the strains recently detected in some European countries (cluster A).

It is noteworthy, that despite its high antigenic heterogeneity and the capability to rapidly develop antimicrobial resistance, *M. iowae* showed overall relatively low genetic variability based on the developed and existing typing methods. Therefore, the experienced high variability and adaptability of *M. iowae* strains are suggested to be influenced at another level, potentially involving gene expression or post-translational modifications of the proteins. In *M. hyopneumoniae* post-translational modifications are relevant and occur frequently in a wide range of functional proteins (Betlach et al., 2019). Moreover, in *M. fermentans,* the post-translational processing increases the complexity of lipoproteins

and the surface antigenic variation within species (Calcutt et al., 1999). It is likely, that such mechanisms play a significant role in generating surface diversity and antigenic heterogeneity in *M. iowae* as well.

5. Summary

The MLVA developed in this study can serve as a proper replacement for the existing MLST method using basic laboratory equipment. The cgMLST demonstrates superior discriminatory power in comparison to the conventional molecular typing approaches; however, the isolation of the pathogen is inevitable for this method. The applied assays consistently distinguished the same major clusters among the diverse selection of isolates. This illustrates their suitability for different genetic analyses and their ability to provide congruent results.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109909.

References

Aycardi, E.R., Anderson, D.P., Hanson, R.P., 1971. Classification of avian mycoplasmas by gel-diffusion and growth-inhibition tests. Avian Dis. 15, 434. https://doi.org/10.2307/1588720.

Bali, K., Bálint, Á., Farsang, A., Marton, S., Nagy, B., Kaszab, E., Belák, S., Palya, V., Bányai, K., 2021. Recombination events shape the genomic evolution of infectious Bronchitis Virus in Europe. Viruses 13, 535. https://doi.org/10.3390/v13040535.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V. M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477. https://doi.org/10.1089/cmb.2012.0021.

Bekő, K., Kreizinger, Z., Sulyok, K.M., Kovács, Á.B., Grózner, D., Catania, S., Bradbury, J., Lysnyansky, I., Olaogun, O.M., Czanik, B., Ellakany, H., Gyuranecz, M., 2019. Genotyping *Mycoplasma gallisepticum* by multilocus sequence typing. Vet. Microbiol 231, 191–196. https://doi.org/10.1016/j.vetmic.2019.03.016.

Benson, G., 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 27, 573–580. https://doi.org/10.1093/nar/27.2.573.

Betlach, A.M., Maes, D., Garza-Moreno, L., Tamiozzo, P., Sibila, M., Haesebrouck, F., Segalés, J., Pieters, M., 2019. *Mycoplasma hyopneumoniae* variability: Current trends and proposed terminology for genomic classification. Transbound. Emerg. Dis. 66, 1840–1854. https://doi.org/10.1111/tbed.13233.

Bottinelli, M., Stefani, E., Matucci, A., Dal Prà, M., Capello, K., Zotti, A., Catania, S., 2021. Isolation of *Mycoplasma iowae* in turkey flocks with skeletal abnormalities: a retrospective study. Avian Pathol. 50, 277–284. https://doi.org/10.1080/03079457.2021.1914816.

Buni, D., Udvari, L., Földi, D., Belecz, N., Yvon, C., Bradbury, J., Catania, S., Lysnyansky, I., Kovács, L., Gyuranecz, M., Kreizinger, Z., 2022. In vitro susceptibility of *Mycoplasma iowae* isolates to antimicrobial agents. Avian Pathol. 51, 374–380. https://doi.org/10.1080/03079457.2022.2072271.

Calcutt, M.J., Kim, M.F., Karpas, A.B., Mühlradt, P.F., Wise, K.S., 1999. Differential posttranslational processing confers intraspecies variation of a major surface lipoprotein and a macrophage-activating lipopeptide of *Mycoplasma fermentans*. Infect. Immun. 67, 760–771. https://doi.org/10.1128/IAI.67.2.760-771.1999.

Catania, S., Gobbo, F., Bilato, D., Fincato, A., Battanolli, G., Iob, L., 2012. Isolation of *Mycoplasma iowae* in commercial turkey flocks. Vet. Rec. 170, 107–108. https://doi.org/10.1136/vr.e645.

Catania, S., Gobbo, F., Rodio, S., Fincato, A., Qualtieri, K., Santone, C., Nicholas, R.A.J., 2014. First isolation of *Mycoplasma iowae* in grey partridge flocks. Avian Dis. 58, 323–325. https://doi.org/10.1637/10661-091113.

Darling, A.C.E., Mau, B., Blattner, F.R., Perna, N.T., 2004. Mauve: Multiple alignment of conserved genomic sequence with rearrangements. Genome Res 14, 1394–1403. https://doi.org/10.1101/gr.2289704.

Deneke, C., Uelze, L., Brendebach, H., Tausch, S.H., Malorny, B., 2021. Decentralized investigation of bacterial outbreaks based on hashed cgMLST. Front Microbiol 12. https://doi.org/10.3389/fmicb.2021.649517.

Fan, H.H., Kleven, S.H., Jackwood, M.W., 1995. Studies of intraspecies heterogeneity of Mycoplasma synoviae, M. meleagridis, and M. iowae with arbitrarily primed polymerase chain reaction. Avian Dis. 39, 766. https://doi.org/10.2307/1592413.

Gautier-Bouchardon, A.V., Reinhardt, A.K., Kobisch, M., Kempf, I., 2002. In vitro development of resistance to enrofloxacin, erythromycin, tylosin, tiamulin and oxytetracycline in *Mycoplasma gallisepticum*, *Mycoplasma iowae* and *Mycoplasma synoviae*. Vet. Microbiol 88, 47–58. https://doi.org/10.1016/S0378-1135(02)00087-1.

Ghanem, M., El-Gazzar, M., 2018. Development of *Mycoplasma synoviae* (MS) core genome multilocus sequence typing (cgMLST) scheme. Vet. Microbiol 218, 84–89. https://doi.org/10.1016/j.vetmic.2018.03.021.

Ghanem, M., El-Gazzar, M., 2016. Development of multilocus sequence typing (MLST) assay for *Mycoplasma iowae*. Vet. Microbiol 195, 2–8. https://doi.org/10.1016/j.vetmic.2016.08.013.

Ghanem, M., Wang, L., Zhang, Y., Edwards, S., Lu, A., Ley, D., El-Gazzar, M., 2018. Core genome multilocus sequence typing: a standardized approach for molecular typing of *Mycoplasma gallisepticum*. J. Clin. Microbiol. 56 https://doi.org/10.1128/JCM.01145-17.

Grózner, D., Kovács, Á.B., Wehmann, E., Kreizinger, Z., Bekő, K., Mitter, A., Sawicka, A., Jánosi, S., Tomczyk, G., Morrow, C.J., Bányai, K., Gyuranecz, M., 2021. Multilocus sequence typing of the goose pathogen *Mycoplasma anserisalpingitidis*. Vet. Microbiol. 254, 108972 https://doi.org/10.1016/j.vetmic.2020.108972.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

Kempf, I., Blanchard, A., Gesbert, F., Guittet, M., Bennejean, G., 1994. Comparison of antigenic and pathogenic properties of *Mycoplasma iowae* strains and development of a PCR-based detection assay. Res Vet. Sci. 56, 179–185. https://doi.org/10.1016/0034-5288(94)90102-3.

Kolmogorov, M., Yuan, J., Lin, Y., Pevzner, P.A., 2019. Assembly of long, error-prone reads using repeat graphs. Nat. Biotechnol. 37, 540–546. https://doi.org/10.1038/s41587-019-0072-8.

Kovács, Á.B., Kreizinger, Z., Forró, B., Grózner, D., Mitter, A., Marton, S., Bali, K., Sawicka, A., Tomczyk, G., Bányai, K., Gyuranecz, M., 2020. The core genome multilocus sequence typing of *Mycoplasma anserisalpingitidis*. BMC Genom. 21, 403. https://doi.org/10.1186/s12864-020-06817-2.

Kreizinger, Z., Sulyok, K.M., Bekő, K., Kovács, Á.B., Grózner, D., Felde, O., Marton, S., Bányai, K., Catania, S., Benčina, D., Gyuranecz, M., 2018. Genotyping *Mycoplasma synoviae*: Development of a multi-locus variable number of tandem-repeats analysis and comparison with current molecular typing methods. Vet. Microbiol 226, 41–49. https://doi.org/10.1016/j.vetmic.2018.10.012.

Matucci, A., Stefani, E., Gastaldelli, M., Rossi, I., De Grandi, G., Gyuranecz, M., Catania, S., 2020. Molecular Differentiation of *Mycoplasma gallisepticum* outbreaks: a last decade study on Italian farms using GTS and MLST. Vaccines 8, 665. https://doi.org/10.3390/vaccines8040665.

Nadon, C.A., Trees, E., Ng, L.K., Møller Nielsen, E., Reimer, A., Maxwell, N., Kubota, K.A., Gerner-Smidt, P., the MLVA Harmonization Working Group, C., 2013. Development and application of MLVA methods as a tool for inter-laboratory surveillance. Eurosurveillance 18. https://doi.org/10.2807/1560-7917.ES2013.18.35.20565.

Nemes, C., Schvarcz, C., Simonyai, E., Turbók, J., Yvon, C., Grózner, D., Gyuranecz, M., 2019. *Mycoplasma iowae* infection in a commercial turkey farm: case report. Magy. Allatorvosok Lapja 141, 589–596.

Panangala, V.S., Gresham, M.M., Morsy, M.A., 1992. Antigenic heterogeneity in *Mycoplasma iowae* demonstrated with monoclonal antibodies. Avian Dis. 36, 108. https://doi.org/10.2307/1591724.

Raviv, Z., Kleven, S.H., 2009. The development of diagnostic real-time Taqman PCRs for the four pathogenic avian mycoplasmas. Avian Dis. 53, 103–107. https://doi.org/10.1637/8469-091508-Reg.1.

Severiano, A., Pinto, F.R., Ramirez, M., Carriço, J.A., 2011. Adjusted Wallace coefficient as a measure of congruence between typing methods. J. Clin. Microbiol 49, 3997–4000. https://doi.org/10.1128/JCM.00624-11.

Silva, M., Machado, M.P., Silva, D.N., Rossi, M., Moran-Gilad, J., Santos, S., Ramirez, M., Carriço, J.A., 2018. chewBBACA: a complete suite for gene-by-gene schema creation and strain identification. Micro Genom. 4. https://doi.org/10.1099/mgen.0.000166.

Staroscik, A., 2004. dsDNA Copy Number Calculator. URI Genomics & Sequencing Center.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38, 3022–3027. https://doi.org/10.1093/molbev/msab120.

Wood, B.J., Wilson, S.J., 2013. *Mycoplasma iowae* in turkeys (*Meleagris gallopavo*). Worlds Poult. Sci. J. 69, 909–916. https://doi.org/10.1017/S0043933913000913.

Zhao, S., Yamamoto, R., 1989. Heterogeneity of *Mycoplasma iowae* determined by restriction enzyme analysis. J. Vet. Diagn. Investig. 1, 165–169. https://doi.org/10.1177/104063878900100214.

Zhou, Z., Alikhan, N.-F., Sergeant, M.J., Luhmann, N., Vaz, C., Francisco, A.P., Carriço, J. A., Achtman, M., 2018. GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. Genome Res. 28, 1395–1404. https://doi.org/10.1101/gr.232397.117.