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Multilocus sequence typing schemes for the emerging swine pathogen *Mycoplasma hyosynoviae*

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ABSTRACT

Mycoplasma (*M.*) *hyosynoviae* is a commensal of the upper respiratory tract in swine, which has the potential to spread systemically, usually resulting in arthritis in fattening pigs and gilts. To date, very little is known about the epidemiology of *M. hyosynoviae,* mainly due to a lack of suitable typing methods. Therefore, this study aimed to develop both a conventional multi locus sequence typing (MLST) and a core genome (cg) MLST scheme. The development of the cgMLST was based on whole genome sequences of 64 strains isolated from pigs and wild boars during routine diagnostics as well as nine publicly available genomes. A cgMLST scheme containing 390 target genes was established using the Ridom© SeqSphere+ software. Using this scheme as a foundation, seven housekeeping genes were selected for conventional MLST based on their capability to reflect genome wide relatedness and subsequently, all 73 strains were typed by applying both methods. Core genome MLST results revealed a high diversity of the studied strain population and less than 100 allele differences between epidemiologically unrelated strains were only detected for four isolates from the US. On the other hand, seven clonal clusters ($\leq$ 12 allele differences) comprising 20 isolates were identified. Comparison of the two typing methods resulted in highly congruent phylogenetic trees and an Adjusted Rand Coefficient of 0.893, while cgMLST showed marginally higher resolution when comparing closely related isolates, indicated by a slightly higher Simpson's ID (0.992) than conventional MLST (Simpson's ID = 0.990). Overall, both methods seem well suited for epidemiological analyses for scientific as well as diagnostic purposes. While MLST is faster and cheaper, cgMLST can be used to further differentiate closely related isolates.

1. Introduction

Mycoplasma (*M.*) *hyosynoviae* (homotypic synonym *Metamycoplasma hyosynoviae*) is a porcine *Mycoplasma* species with worldwide distribution (Palzer et al., 2020). It was firstly described by Ross and Karmon (1970) and is primarily found as harmless commensal in the nasal cavity and the tonsils of pigs (Gomes Neto, 2012). After systemic spread, *M. hyosynoviae* exhibits an affinity to joint tissue (Kobisch and Friis, 1996; Hagedorn-Olsen et al., 1999) and causes acute non-purulent arthritis in growing and finishing pigs (Lauritsen et al., 2017; Pieters and Maes, 2019; Palzer et al., 2020).

Even though the prevalence of *M. hyosynoviae* infections in pigs seems to be on the rise in recent years, little is known about the epidemiology of this porcine pathogen (Schwartz et al., 2014; Palzer et al., 2020). While early colonization of suckling piglets has been confirmed, transmission during the suckling period is less frequent than with other porcine mycoplasmas (Hagedorn-Olsen et al., 1999; Schwartz et al., 2014; Roos et al., 2019). Instead, most piglets are colonized after weaning with a prevalence peaking between 10 and 16 weeks of age (Schwartz et al., 2014; Roos et al., 2019). Similar to *M. hyorhinis*,

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systemic spread only occurs in a subpopulation of animals colonized by *M. hyosynoviae*, usually resulting in synovial lesions and the development of clinical signs such as increased production of joint fluid and lameness (Pieters and Maes, 2019).

Very little information is available about the epidemiology of *M. hyosynoviae*, and epidemiological investigations are limited as no suitable typing methods have been described for this pathogen so far. For several *Mycoplasma* species, including the porcine species *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* multi locus sequence typing (MLST) schemes have been developed and published at pubMLST (Mayor et al., 2008; Tocqueville et al., 2014; Fourour et al., 2019). Recently, core genome (cg) MLST schemes have been established for *M. bovis* in cattle (Kinnear et al., 2021; Menghwar et al., 2022), *M. gallisepticum*, *M. synoviae*, and *M. anserisalpingitidis* in poultry (Ghanem and El-Gazzar, 2018; Kovács et al., 2020) and for the porcine species *M. hyorhinis* (Bünger et al., 2021). While cgMLST usually results in higher resolution than conventional MLST, it is more time-consuming and expensive limiting its suitability for routine diagnostics. For epidemiologic investigations, however, the increased discriminatory power has shown to be useful for differentiation of closely related strains (Ghanem and El-Gazzar, 2018; Bünger et al., 2021; Kinnear et al., 2021; Menghwar et al., 2022).

In order to facilitate epidemiological research on *M. hyosynoviae* and to gain insights into the pathogen's population structure, we developed two novel sequence-typing schemes based on analyzing 73 *M. hyosynoviae* strains. First, a cgMLST scheme utilizing 390 target genes was developed and subsequently, a conventional MLST scheme targeting seven housekeeping genes was established, and a web-accessible database set up for the *M. hyosynoviae* MLST scheme at https://pubmlst.org/ (Jolley et al., 2018).

2. Materials & methods

2.1. M. hyosynoviae strains and whole genome sequencing

In total, 73 *M. hyosynoviae* strains were fully analyzed in the study including 38 strains isolated from pigs (n = 30) and wild boars (n = 8) in Austria, 22 and one strain recovered from pigs and a wild boar in Germany, respectively, and three strains from pigs in Norway. Further, eight *M. hyosynoviae* strains from North America (including type strain ATCC 25591^T) (Bumgardner et al., 2014) and one from Denmark with publicly available whole genome sequences (Table S1) were included. Seven additional strains isolated from pigs in Germany, which appeared to be non-viable after shipment to Austria, were only tested by conventional MLST (Table S1). All cultured strains (n = 64) were obtained from diagnostic samples taken from different body sites (eye, nasal cavity, trachea, lung, lymph node, serosa, joint) submitted for microbiological investigation between 2002 and 2023. All pigs showed some form of clinical disease and/ or pathologic lesions primarily (arthritis) or secondarily (pneumonia, serositis, conjunctivitis) associated with *M. hyosynoviae* whereas in wild boar the pathogen was almost exclusively isolated from pneumonic lungs and pulmonary lymph nodes (Table S1). The 22 cultured strains from domestic pigs in Germany originated from eight different farms, while the 30 Austrian *M. hyosynoviae* pig isolates came from 29 farms, and the three Norwegian isolates from one premise. Multiple *M. hyosynoviae* strains were derived from six farms (n = 23 strains; 18 strains from four German farms, three and two strains from one Norwegian and one Austrian farm, respectively) and in one German farm three isolates were derived from one individual pig (B3J20G, B4J20G, B5J20G; Table S1). All cultured *M. hyosynoviae* strains were identified at the species level using MALDI-TOF mass spectrometry (MS) as described previously (Spergser et al., 2019), and stored at −80 °C until further investigation.

For whole genome sequencing (WGS), *M. hyosynoviae* stock cultures were thawed, diluted 1:100, and plated onto SP4 agar (Ramírez et al., 1997) before incubation at 37 °C under 5 % CO_2 atmosphere for up to 7 days (Bünger et al., 2021). Subsequently, an isolated colony was picked and cultured in 15 mL SP4 medium until a color change of the medium from orange to pink occurred (based on alkalinization resulting from arginine hydrolysis). Cultures were pelleted by centrifugation at 20,000 x g for 10 min and DNA was extracted using MagAttract® HMW DNA Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany). Quantity and quality of extracted DNA were determined by Qubit™ 4 fluorometer and NanoDrop™ 2000 (both Thermo Scientific, Vienna, Austria). Whole genome sequencing was performed on libraries prepared by the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, USA), and an Illumina MiSeq or MiniSeq platform (Illumina, San Diego, USA) was applied for paired-end sequencing. Raw reads were quality checked, trimmed, and *de novo* assembled using SPAdes 3.10. (Bankevich et al., 2012). Three *M. hyosynoviae* strains (B1J20G, B4J20G, B7E20G) were additionally sequenced on a Nanopore MinION device (Oxford Nanopore Technologies, Oxford, UK) and Illumina short reads along with Nanopore long reads were hybrid assembled utilizing the Unicycler pipeline 0.4.8 with default settings (Wick et al., 2017). Completed and draft genomes of all 64 cultured *M. hyosynoviae* strains were deposited at Genbank and are available under the accession numbers provided in Table S1.

2.2. Core genome MLST

A cgMLST scheme for *M. hyosynoviae* was developed using Ridom© SeqSphere + version 7.0 (Ridom© GmbH, Münster, Germany; https://www.ridom.de/seqsphere/) as described previously for *M. hyorhinis* (Bünger et al., 2021). In brief, the genome of strain B1J20G finished in this study (NZ_CP101127), and 12 query genomes were utilized for the development of an *ad hoc* cgMLST scheme. Query genomes were selected based on their origin including strains from different geographic locations, years of isolation, body sites, and hosts (Table S2). As first step in cgMLST development, a target gene set appropriate for cgMLST was defined using the cgMLST Target Definer tool that filtered the reference genome (NZ_CP101127) to exclude unfit gene targets (Bünger et al., 2021). Next, the 12 query genomes were blasted pairwise against the reference genome's target gene set to select shared targets with sequence identities of > 90 % and an overall overlap constituting the final targets of the cgMLST scheme. For evaluation purposes, the remaining 60 genomes (Table S1, S2) were used to determine the percentage of cgMLST targets present in the *M. hyosynoviae* strain cohort. Eventually, all 73 *M. hyosynoviae* strains were typed by the newly developed cgMLST scheme to define individual allelic profiles that were utilized to construct a minimal spanning tree (MST). In addition, allelic distances between closely related *M. hyosynoviae* strains were determined by comparing 23 isolates from six farms (> 1 isolate per farm) and the threshold for clonal cluster definition was assessed based on results of the cgMLST MST. Furthermore, a phylogenetic tree was calculated using the neighbor joining algorithm implemented in the SeqSphere + software and visualized by iTOL (Letunic and Bork, 2019).

2.3. Conventional MLST

For the development of a conventional MLST, housekeeping genes used in previously described *Mycoplasma* MLST schemes (Jolley et al., 2018) were screened for even distribution in the *M. hyosynoviae* B1J20G genome, their diversity among the strains analyzed, and their capability to reflect genome-wide relatedness as shown by cgMLST. Using this approach, the seven housekeeping genes *dnaA* (chromosomal replication initiator protein), *ftsY* (signal recognition particle receptor), *gyrB* (DNA gyrase subunit B), *recA* (recombinase A), *rpoB* (DNA-directed RNA polymerase subunit beta), *uvrA* (excinuclease ABC subunit A), and *fusA* (elongation factor G) appeared to be most suitable for the establishment of a conventional MLST scheme. Primers flanking the variable regions of the selected housekeeping genes were then designed using Primer 3 (version 2.3.7) plugin in Geneious Prime® 2022.1 (Biomatters Ltd., New

Zealand), and the specificity of the primers was verified by BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer sequences, fragment positions in the genome of B1J20G and amplicon sizes are listed in Table 1. For PCR evaluation, each housekeeping gene fragment was amplified from 20 selected *M. hyosynoviae* strains (Table S3), and amplicons were Sanger sequenced at LGC Genomics Berlin, Germany. DNA extracts from *M. hyopneumoniae* J^T, *M. flocculare* Ms42^T, *M. hyorhinis* BTS-7^T, and *M. hyopharyngis* H3–6B F^T were applied as negative controls. The PCR mixtures contained 12.5 µL One*Taq*® Quick-Load® 2x Master Mix with Standard Buffer (1x containing 25 units/ mL One*Taq*® DNA Polymerase, 1.8 mM $MgCl_2$, and 0.2 mM dNTPs) (New England Biolabs® GmbH), 0.5 µL of each primer (10 pmol/µL), 9 µL ddH_2O, and 2.5 µL DNA template yielding a total volume of 25 µL. The PCR was performed on a Mastercycler® nexus PCR thermocycler (Eppendorf Austria GmbH) with the following cycling conditions: 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. For the remaining *M. hyosynoviae* strains, housekeeping gene sequences were extracted from WGS using Geneious Prime® 2022.1. Sequences were then aligned by ClustalW in the MegaX software (Kumar et al., 2018) and consecutive numbers (starting with *M. hyosynoviae* type strain ATCC25591^T) assigned to each new allelic type or allele combination (ST) with identical sequences or combinations being assigned the same allele or ST number. In addition, sequences of the housekeeping gene fragments were concatenated, and a phylogenetic tree constructed using the Maximum Likelihood method and Hasegawa-Kishino-Yano substitution model with bootstrapping (1000 replications) in MegaX (Fig. 3; Kumar et al., 2018).

For both cgMLST and conventional MLST schemes the discriminatory power was determined and compared based on the Simpson's index of diversity (ID) (Hunter and Gaston, 1988) applying the online tool Comparing Partitions (http://www.comparingpartitions.info/) using default parameters. To assess the concordance between cgMLST and conventional MLST Adjusted Rand coefficients (Hubert and Arabie, 1985) were also calculated by Comparing Partitions.

Table 1
Primer sequences, genome positions, and amplicon sizes of seven housekeeping genes targeted by MLST.

Gene	Primer sequences 5′-3′	Genome position (B1J20G)	Amplicon size
dnaA	Forward: TTTCTTCAATAGAACGTGCAATTCA Reverse: TGAGCATATTGTTGAATGTCATCAA	228-727	499 bp
ftsY	Forward: CGTTGAGGGTTTATCTAGATCAAACA Reverse: TTTTGCAATCTACCAGCAGTATCAA	141,675-142,260	585 bp
gyrB	Forward: ACGTGGTAAGGTAATTAATGCTGAA Reverse: TGGATCCATTTCACCAAGACCTTTA	248,429-248,823	394 bp
recA	Forward 1: TGTTGATTGTTTGTCGAAAACAGGA Reverse: TCAGCAAGTTTTGAAATTCCTTTTGA	295,484-295,919	435 bp
rpoB	Forward: TAACTGCTATTGAAGAAATGGGTGC Reverse 1: TTTTTACCAAGTGCAAGTTCACCAT	440,773-441,365	592 bp
uvrA	Forward: TGGGTAAATTCATTGTAGTTTCAGGA Reverse: ACATCAGGTAAAAAGTGCATTTCAA	725,188-725,588	400 bp
fusA	Forward: TGCTGTTGTTGATTACTTACCTTCG Reverse: CCTGATTGTTTCTTGTGCATACCTT	749,839-750,520	681 bp

3. Results

All cultured *M. hyosynoviae* strains (n = 64) were successfully sequenced using an Illumina MiSeq or MiniSeq platform. Resulting raw reads were assembled *de novo* generating assemblies with a mean size of 858.666 Kb representing full genomes of *M. hyosynoviae*, a mean number of 51 contigs (>500 bases), and an average calculated coverage of 82. Genome assemblies were deposited at GenBank databases (number of contigs and average coverage of each genome as well as accession numbers are presented in Table S1). For three *M. hyosynoviae* strains (B1J20G, B4J20G, B7E20G) completed genomes were generated by hybrid assembly of Nanopore and Illumina reads resulting in circular genomes (NZ_CP101127, NZ_CP101128, NZ_CP101129) with genome sizes of 889.181 (320 x coverage), 880.327 (420 x coverage), and 906.150 Kb (450 x coverage) for B1J20G, B4J20G, and B7E20G, respectively (Table S1).

3.1. Development of a M. hyosynoviae cgMLST scheme

From the completed genome of strain B1J20G (NZ_CP101127) containing 695 coding gene sequences, a reference task template was generated that retained a total of 641 genes after filtering. By using 12 query genomes, a cgMLST scheme with 390 target genes (435,206 bases) was defined, covering 49 % of the reference genome sequence. The number of accessory genes that can be used for top-down analyses was 251 (318,799 bases), representing 35.9 % of the reference genome. Core target genes defined are listed in Table S2. For cgMLST evaluation, WGS of the remaining 60 *M. hyosynoviae* strains were loaded into SeqSphere + and typed according to the 390 cgMLST target scheme. At least 95 % (mean 98.1 % ± 1.24 standard deviation for all genomes) cgMLST target genes were found in WGS used for evaluation. The average number of alleles for 390 targets was 22 alleles ranging from three to 55 alleles. To specify clonal cluster distances, first a subset of 23 isolates from six farms (four German farms, one Austrian, and one Norwegian farm) was used. Five of these isolates (3432_4J15G, A1608804_005J20G, A16008804_07J20G, B1J20G, B7E20G), however, were excluded from cluster threshold determination as they showed numbers of allele differences (≥ 34 allele differences, > 8.7 % of core target genes) uncommon for clonal cluster formation (Fig. 1; Ghanem and El-Gazzar, 2018; Bünger et al., 2021). The maximum number of allele differences observed among the remaining 18 strains was 12 alleles, which was set as threshold for distinct clonal clusters. Based on this threshold, seven different clonal clusters comprising 20 *M. hyosynoviae* strains were identified (Figs. 1, 2).

3.2. Typing of M. hyosynoviae strains using cgMLST

The newly developed cgMLST scheme was tested on all 73 strains to gain insights into the population structure of *M. hyosynoviae*, and to evaluate the genome-wide relatedness of the strains included in the study. Allelic profiles for all strains are listed in Table S2. Overall, results of cgMLST showed a high degree of diversity among epidemiologically unrelated *M. hyosynoviae* strains illustrated by a high number of allele differences between these strains exhibiting allele differences in more than half of the 390 analyzed target genes (Fig. 1, Fig. 2). Regarding the origin of the studied strain population, the strains from the US and Canada (excluding type strain ATCC 25591^T) were separated clearly from the European isolates (Fig. 1). Within the European isolates, the single Danish (M60) and three Norwegian strains (SN1J23N, SN2J23N, SN3J23N, Cluster 4) were separated from the Austrian strain cohort, while the German strains were mostly contained in three semi-separated branches of the MST (Fig. 1). Wild boar isolates included in this study were not forming their own sub-branch but were dispersed all over the tree. In addition, high numbers of allele differences were evident between wild boar and closest related pig isolates indicating that intra-specific pathogen transmission between wild and domesticated pig

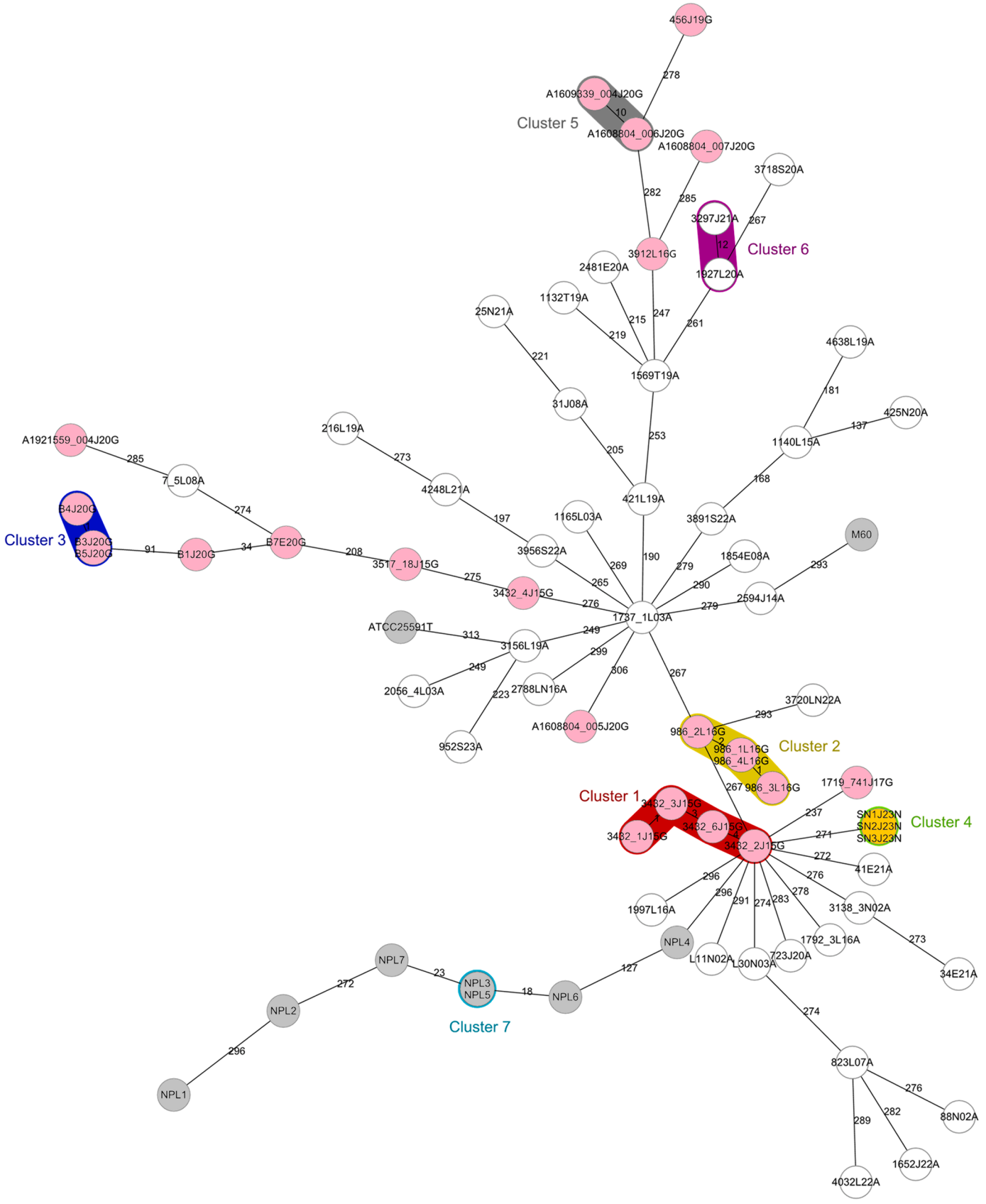

Fig. 1. Minimum spanning tree constructed from allelic profiles (390 core genome targets) of 73 *M. hyosynoviae* strains using the pairwise ignoring-missing values option. The seven distinct clonal clusters with ≤ 12 allele differences are color coded. The number on connecting lines illustrates the number of differing alleles in a pair-wise comparison. The color of the circles indicates strain origin (grey = publicly available genome (USA, Canada, Denmark); orange = Norway; pink = Germany, white = Austria).

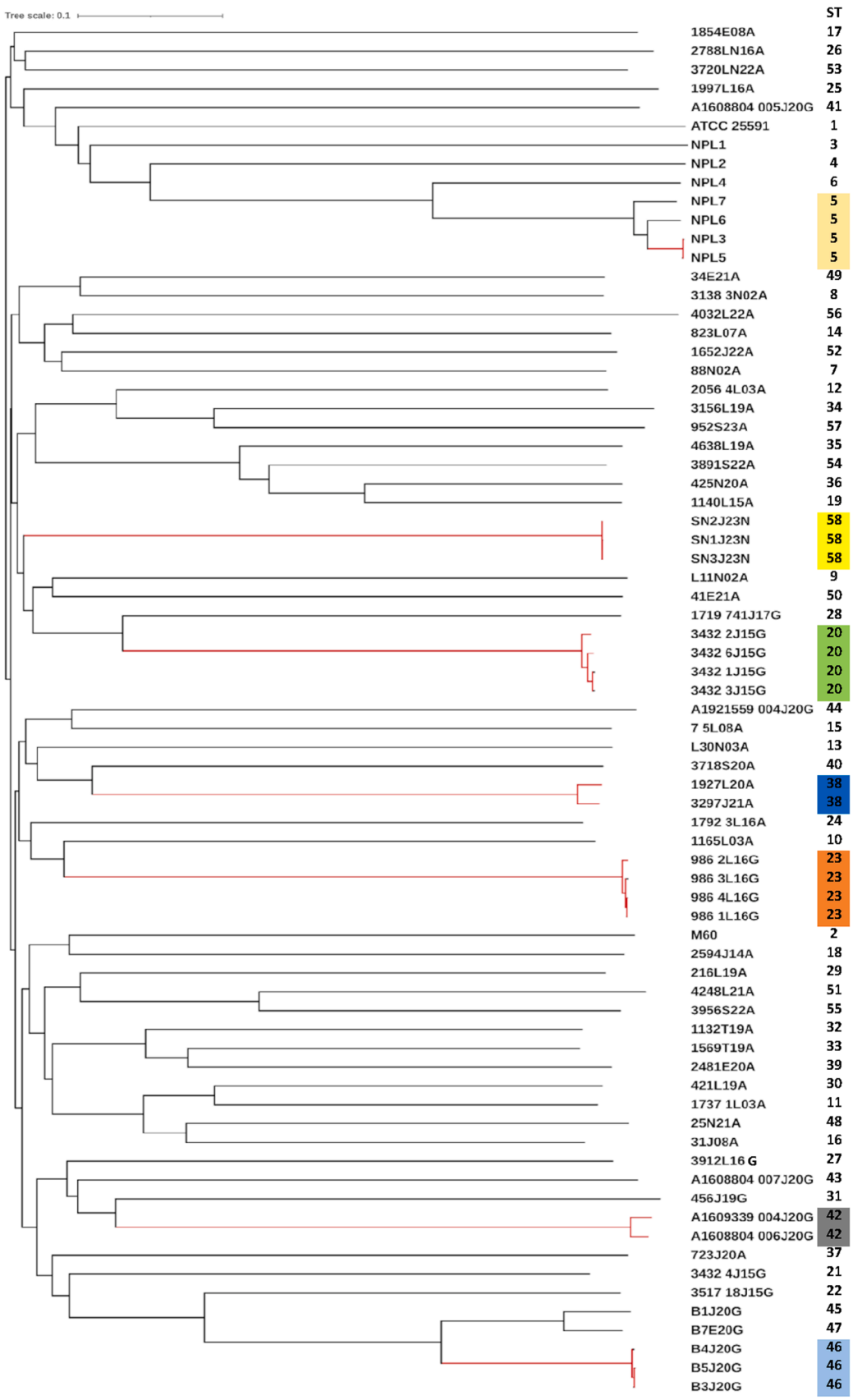

Fig. 2. Phylogenetic tree built from allelic profiles (390 core genome targets) of 73 *M. hyosynoviae* strains using the neighbor joining algorithm and the pairwise ignoring-missing-values option. Clades of clonal clusters are highlighted in red. Conventional MLST sequence types are depicted opposed to the tree. Visualization was realized using iTOL.

populations is rather unlikely. Altogether, cgMLST typed the 73 *M. hyosynoviae* strains into 60 different sequence types (STs) based on > 12 allele differences (threshold for clonal clusters) resulting in a Simpson's ID of 0.992.

3.3. Development and application of a conventional M. hyosynoviae MLST scheme

Gene fragments of the selected housekeeping genes *dnaA*, *ftsY*, *gyrB*, *rpoB*, *uvrA*, *recA* and *fusA* were successfully amplified from 20 selected *M. hyosynoviae* strains (Table S3) followed by Sanger sequencing. No cross-reactions were observed when DNA from other swine mycoplasmas (*M. hyopneumoniae*, *M. flocculare*, *M. hyorhinis*, *M. hyopharyngis*) were used as negative controls. In addition, housekeeping gene fragments were extracted from WGS of all 73 *M. hyosynoviae* strains and Sanger sequences compared with genome extracted sequences of 13 *M. hyosynoviae* strains (from seven strains no WGS were available, Table S1, S3) which were shown to be identical. Finally, the seven housekeeping gene fragments were concatenated (3586 bp) and a phylogenetic tree was constructed applying the Maximum Likelihood algorithm and the Hasegawa-Kishino-Yano substitution model in MegaX (Fig. 3). The analyzed 80 *M. hyosynoviae* strains were typed into 63 different STs (Simpson's ID of 0.991). If only considering the 73 strains which were also typed by cgMLST, the Simpson's ID was 0.990. Allelic profiles and STs are listed in Table S3. When STs obtained from MLST were compared to cgMLST results and clonal cluster formation, a slightly lower resolution capability of the conventional MLST scheme was evident. However, the Adjusted Rand coefficient defining the proportion of congruence between the typing methods used was excellent (0.893) indicating high concordance between the utilized typing methods.

An online database for the conventional MLST scheme, comprising 132 allele sequences (*dnaA* n = 17, *ftsY* n = 22, *gyrB* n = 15, *recA* n = 20, *rpoB* n = 19, *uvrA* n = 22, and *fusA* n = 17), 63 STs, and 80 *M. hyosynoviae* strains, was developed (Jolly et al., 2018) and is publicly available at https://pubmlst.org/organisms/mycoplasma-hyosynoviae/.

4. Discussion

This study describes the development of two novel allele-based sequence typing methods for *M. hyosynoviae* which were subsequently utilized to type European and North American field strains. Similar to previous studies, we found cgMLST to have slightly better discriminatory power than conventional MLST when comparing closely related targets (Ghanem et al., 2018; Bünger et al., 2021; Kinnear et al., 2021). However, the selection of seven target genes from whole genome sequences of 73 strains led to the development of a MLST scheme which possessed sufficient resolution for many applications. Further, comparison of MLST and cgMLST results (Fig. 2) proved that the novel MLST scheme is properly reflecting genomic relationship between the isolates in this study, affirmed by the high congruence between the two methods. For the novel cgMLST scheme, the threshold for clonal cluster was set at ≤ 12 allele differences, very close to the cluster thresholds set for other *Mycoplasma* cgMLST schemes (Ghanem et al., 2018; Bünger et al., 2021; Menghwar et al., 2022). Based on the strains analyzed within this study and taking the nucleotide variability after multiple passages into account (Ghanem and El-Gazzar, 2018), ≤ 12 allele differences should be a solid value to ensure that only clonally related isolates are typed into a clonal cluster.

Interestingly, most epidemiologically unrelated isolates differed in > 50 % of the 390 target genes, indicating a highly diverse *M. hyosynoviae* population expressing limited clonality. When compared to cgMLST results of *M. hyorhinis* (Bünger et al., 2021), another ubiquitous porcine pathogen, the differences between *M. hyosynoviae* isolates are much greater on average even when only comparing isolates from Austria. While four isolates from the US (NPL3, NPL5, NPL6, NPL7) showed very few allele differences and two of them even formed a clonal cluster (NPL3, NPL5, Cluster 7), these four strains were the only presumably epidemiologically unrelated strains with < 100 allele differences (Fig. 1). On the other hand, two isolates from farm C (1927L20A, 3297J21A) formed a clonal cluster with only 12 allele differences even though they were isolated 15 months apart in the years 2020 and 2021. This indicates that *M. hyosynoviae* subpopulations on individual farms might be stable over time. In contrast, multiple subpopulations can coexist within one farm, as two isolates from farm D (A1609339_004J20G, A1608804_006J20G) formed a clonal cluster, while only being very distantly related to two other strains from the same farm which were isolated at the same time (A1608804_005J20G, A1608804_007J20G). Both observations have previously also been made for *M. hyorhinis* (Bünger et al., 2021), indicating a similar population and subpopulation structure for these two porcine *Mycoplasma* species.

While the American, German, and Austrian strain cohorts mostly grouped in specific branches of the minimum spanning tree (Fig. 1), the overall high number of allele differences regardless of the geographical location, make it almost impossible to conclude on strain origin only based on cgMLST results. Similar findings were made for *M. anserisalpingitidis* (Kovács et al., 2020), while cgMLST analysis of 151 *M. bovis* strains resulted in fewer allele differences for isolates from specific regions and provinces (Menghwar et al., 2022). The high differences between presumably unrelated *M. hyosynoviae* strains might, however, allow for easier detection of related strains, as < 100 allele differences were not regularly observed.

As a final comment, this study mostly contains strains isolated during routine diagnostics from wild boar or from domestic pigs showing some form of clinical disease. Therefore, these strains are not representative of the European or even the German/ Austrian *M. hyosynoviae* populations. To further elucidate and confirm the high diversity found in the strain cohort of this study, it is necessary to investigate a wider sample pool in the future. Therefore, the MLST scheme we developed has been made publicly available on pubMLST.org (Jolley et al., 2018) and sequences, allelic profiles (STs) and information on the 80 strains have been uploaded to the database.

5. Conclusion

In summary, the novel MLST and cgMLST schemes presented in this study allow for accurate sequence typing of *M. hyosynoviae* isolates, representing the first allele-based typing methods for this emerging swine pathogen. The conventional MLST scheme is suitable for most applications in routine diagnostics, while cgMLST might be used for further differentiation in the case of closely related isolates sharing the same sequence type.

Ethics approval

An ethical approval was not required since all samples were taken for diagnostic purposes during clinical examination or at necropsy with the consent of the animal owners.

CRediT authorship contribution statement

Ladinig Andrea: Resources, Writing – review & editing. **Ruppitsch Werner:** Investigation, Methodology. **Teich Klaus:** Resources. **Kübber-Heiss Anna:** Resources. **Hennig-Pauka Isabel:** Resources. **Bünger Moritz:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Blümlinger Michael:** Investigation, Methodology. **Loncaric Igor:** Investigation, Methodology. **Rosel Adriana Cabal:** Investigation, Methodology. **Spergser Joachim:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology,

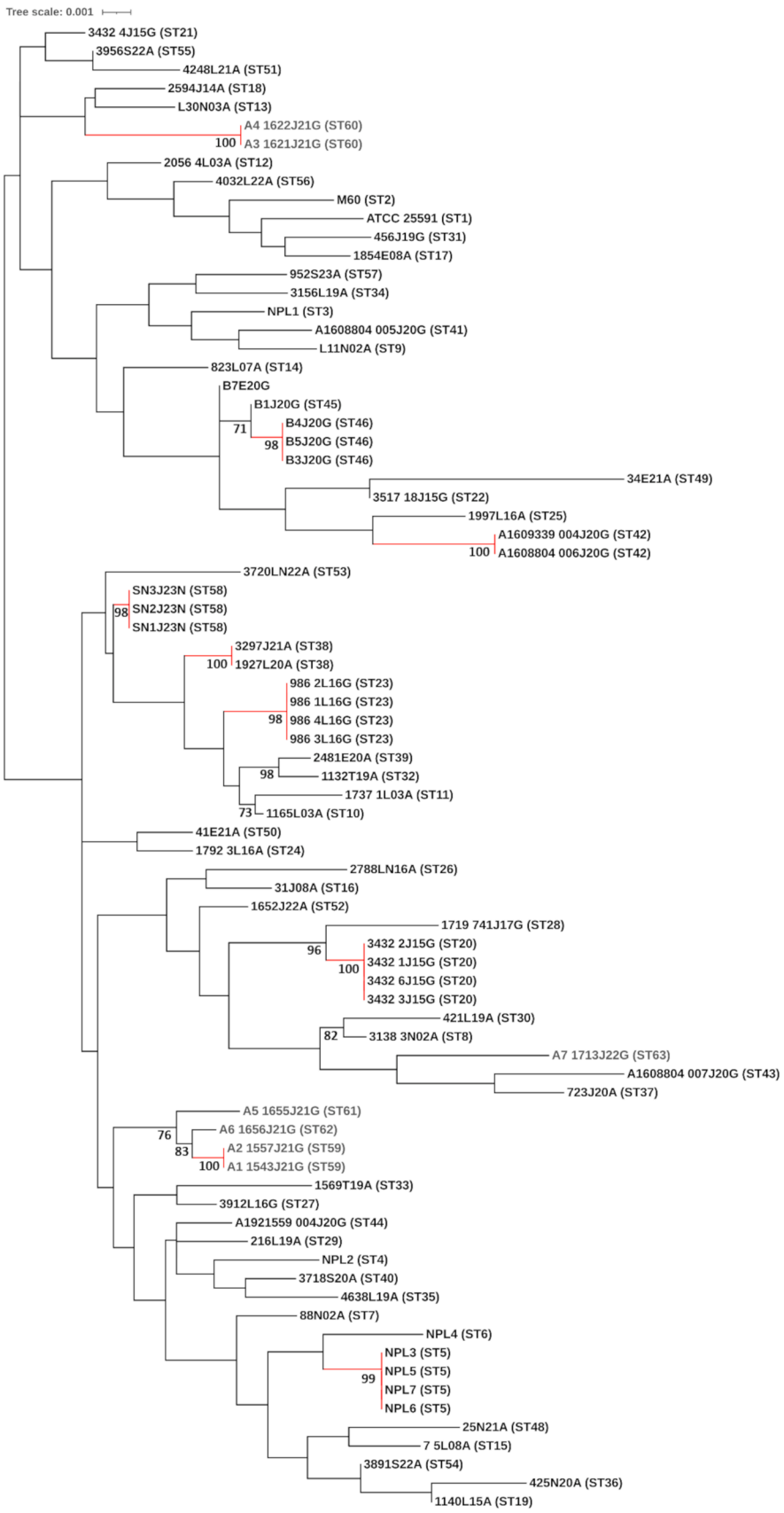

Fig. 3. Phylogenetic tree based on concatenated sequences of the seven housekeeping gene fragments (*dnaA*, *ftsY*, *gyrB*, *recA*, *rpoB*, *uvrA*, *fusA*). The tree was constructed using the Maximum Likelihood method and Hasegawa-Kishino-Yano substitution model with 1000 bootstraps (only bootstrap values > 70 % are presented) in MegaX. Branches of strains belonging to the same ST are highlighted in red. Strains only typed by conventional MLST are in gray.

Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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.2024.109997.

References

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.

Bumgardner, E., Bey, R.F., Kittichotirat, W., Bumgarner, R.E., Lawrence, P.K., 2014. Genome sequences of seven *Mycoplasma hyosynoviae* strains isolated from the joint tissue of infected swine (*Sus scrofa*). Genome Announc. 2, 2–3. https://doi.org/10.1128/genomeA.00552-14.

Bünger, M., Posch, M., Wiesauer, J., Loncaric, I., Cabal Rosel, A., Ruppitsch, W., Ladinig, A., Spergser, J., 2021. A core genome multilocus sequence typing scheme for *Mycoplasma hyorhinis*. Vet. Microbiol. 262, 109249 https://doi.org/10.3389/fmicb.2020.01049.

Fourour, S., Lucas, P., Touzain, F., Tocqueville, V., Gautier-Bouchardon, A.V., Kempf, I., Marois-Créhan, C., 2019. Genomic polymorphism of *Mycoplasma flocculare* revealed by a newly developed multilocus sequence typing scheme. Vet. Microbiol. 237, 108422 https://doi.org/10.1016/j.vetmic.2019.108422.

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.

Gomes Neto, J.C., Gauger, P.C., Strait, E.L., Boyes, N., Madson, D.M., Schwartz, K.J., 2012. *Mycoplasma*-associated arthritis: critical points for diagnosis. J. Swine Health Prod. 20, 82–86.

Hagedorn-Olsen, T., Nielsen, N.C., Friis, N.F., 1999. Induction of arthritis with *Mycoplasma hyosynoviae* in pigs: clinical response and re-isolation of the organism from body fluids and organs. J. Vet. Med. A 46, 317–325. https://doi.org/10.1046/j.1439-0442.1999.00217.x.

Hubert, L., Arabie, P., 1985. Comparing partitions. J. Classif. 193–218.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465–2466. https://doi.org/10.1128/jcm.26.11.2465-2466.1988.

Jolley, K.A., Bray, J.E., Maiden, M.C.J., 2018. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 3, 124. https://doi.org/10.12688/wellcomeopenres.14826.1.

Kinnear, A., Waldner, M., McAllister, T.A., Zaheer, R., Register, K., Jelinski, M., 2021. Application of four genotyping methods to *Mycoplasma bovis* isolates derived from Western Canadian feedlot cattle. J. Clin. Microbiol. 59, e0004421 https://doi.org/10.1128/jcm.00044-21.

Kobisch, M., Friis, N.F., 1996. Swine mycoplasmoses. OIE Rev. Sci. Tech. 15, 1569–1605. https://doi.org/10.20506/rst.15.4.983.

Kovács, Á.B., Kreizinger, Z., Forró, B., Grózner, D., Mitter, A., Marton, S., Bali, K., Sawicka, A., Tomczyk, G., Bányai, K., Gyuranecz, M., Gyuranecz, M., 2020. The core genome multi-locus sequence typing of *Mycoplasma anserisalpingitidis*. BMC Genom. 21 (1), 8. https://doi.org/10.1186/s12864-020-06817-2.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549. https://doi.org/10.1093/molbev/msy096.

Lauritsen, K.T., Hagedorn-Olsen, T., Jungersen, G., Riber, U., Stryhn, H., Friis, N.F., Lind, P., Kristensen, B., 2017. Transfer of maternal immunity to piglets is involved in early protection against *Mycoplasma hyosynoviae* infection. Vet. Immunol. Immunopathol. 183, 22–30. https://doi.org/10.1016/J.VETIMM.2016.12.002.

Letunic, I., Bork, P., 2019. Interactive Tree of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47, 256–259. https://doi.org/10.1093/nar/gkz239.

Mayor, D., Jores, J., Korczak, B.M., Kuhnert, P., 2008. Multilocus sequence typing (MLST) of *Mycoplasma hyopneumoniae*: a diverse pathogen with limited clonality. Vet. Microbiol. 127, 63–72. https://doi.org/10.1016/j.vetmic.2007.08.010.

Menghwar, H., Guo, A., Chen, Y., Lysnyansky, I., Parker, A.M., Prysliak, T., Perez-Casal, J., 2022. A core genome multilocus sequence typing (cgMLST) analysis of *Mycoplasma bovis* isolates. Vet. Microbiol. 273, 109532 https://doi.org/10.1016/j.vetmic.2022.109532.

Palzer, A., Ritzmann, M., Spergser, J., 2020. *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* in pig herds. In: Maes, D., Sibila, M., Pieters, M. (Eds.), Mycoplasmas in Swine. Uitgeverij ACCO, Leuven, pp. 247–265.

Pieters, M., Maes, D., 2019. Mycoplasmosis. In: Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W., Zhang, J. (Eds.), Diseases of Swine, 11th edition. Wiley Blackwell, Hoboken, pp. 863–883.

Ramírez, A.S., González, M., Déniz, S., Fernández, A., Poveda, J.B., 1997. Evaluation of a modified SP-4 medium in the replication of *Mycoplasma* spp. In: Frey, J., Sarris, K. (Eds.), Mycoplasmas of Ruminants: Pathogenicity, Diagnostics, Epidemiology and Molecular Genetics. European Cooperation on Scientific and Technical Research (COST), Luxembourg, pp. 36–39.

Roos, L.R., Nair, M.S., Rendahl, A.K., Pieters, M., 2019. *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* dual detection patterns in dams and piglets. PLoS One 14 (1), 12. https://doi.org/10.1371/journal.pone.0209975.

Ross, R.F., Karmon, J.A., 1970. Heterogeneity among strains of *Mycoplasma granularum* and identification of *Mycoplasma hyosynoviae*, sp. n. J. Bacteriol. 103, 707–713. https://doi.org/10.1128/jb.103.3.707-713.1970.

Schwartz, J., Bruner, L., Evelsizer, B., Konz, B., Rovira, A., Pieters, M., 2014. Dynamics of *Mycoplasma hyosynoviae* detection and clinical - presentation. Am. Assoc. Swine Vet. 115–116.

Spergser, J., Hess, C., Loncaric, I., Ramírez, A.S., 2019. Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. J. Clin. Microbiol. 57, 1–16. https://doi.org/10.1128/JCM.00316-19.

Tocqueville, V., Ferré, S., Nguyen, N.H.P., Kempf, I., Marois-Créhana, C., 2014. Multilocus sequence typing of *Mycoplasma hyorhinis* strains identified by a real-time TaqMan PCR assay. J. Clin. Microbiol. 52, 1664–1671. https://doi.org/10.1128/JCM.03437-13.

Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E., 2017. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput. Biol. 13, 1–22. https://doi.org/10.1371/journal.pcbi.1005595.