

Title: Characterization of the genetic diversity of *Mycoplasma hyorhinis* field isolates by multiple locus variable number of tandem repeats analysis (MLVA) and multi-locus sequence typing (MLST) - NPB #12-044

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Industry Summary:

Mycoplasma hyorhinis is an important pathogenic organism that inhabits the nasal cavity of swine. This bacterium has been associated with a variety of diseases in pigs, where polyserositis and arthritis represent the main and more serious lesions. However, pigs can frequently be asymptomatic nasal carriers. In recent years swine practitioners have noticed an increase in the appearance of *M. hyorhinis*-associated disease cases. Additionally, veterinary diagnostic laboratories have observed similar trends. Data from the Veterinary Diagnostic Laboratory at Iowa State University, showed that in 2010, 37% of infectious arthritis cases were positive for *Mycoplasma* species, compared to 17% in the previous year. Also, the University of Minnesota Veterinary Diagnostic Laboratory reported that 55% of polyserositis and 12% of arthritis cases received tested positive for this pathogen by PCR in 2012. Efforts to control the disease have been hindered by the lack of knowledge on the epidemiology and ecology of *M. hyorhinis*. The understanding of transmission dynamics, identification of the source of infection and virulent strains, and the discovery of vaccine-relevant antigens is essential in disease control programs. There are currently no molecular epidemiological tools available for *M. hyorhinis*. **Therefore the main objective of this study was to develop and validate a multilocus sequence typing (MLST) assay for the characterization of *M. hyorhinis* isolates.**

Thirty-eight *M. hyorhinis* isolates obtained in 2010-2011 from disease pigs, in addition to one reference strain, were utilized in this study. These isolates originated from 11 states, 18 systems, three pig stages and different lesion sites within the pigs. Isolates were cultured in Modified Hayflick's media for 7-14 days followed by DNA extraction. The genome sequences of four *M. hyorhinis* isolates were utilized to identify potential target genes. The classical MLST scheme was modified to target not only housekeeping genes (slowly accumulate nucleotide changes) but also hyper-variable genes (accumulate changes over a short period of time). This modified scheme would potentially allow us to increase the discriminatory power, making this assay highly useful for outbreak

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investigations. After selection of the target genes, primers were designed and PCR amplification was carried out. PCR products were purified and sequenced. The data obtained was checked for quality and trimmed to the appropriate length. Sequences from all isolates within each gene were aligned and dendrograms were constructed from individual genes, as well as, the concatenated sequence of all genes. Within each locus (gene) distinct alleles were assigned arbitrary allele numbers. For each isolate, the sequence type (ST) was defined by the combination of the allele number at each of the loci.

More than 25 genes were evaluated as potential gene targets with varying degrees of success. The final MLST protocol included the following genes: *ung*, *pdhB*, *mtlD*, *p3*, *p95*. Variation within each gene range from 0.5-20%. The number of alleles per gene varied from 3-11, giving rise to 27 sequence types (STs) within the 39 isolates. The dendrogram constructed based upon concatenated gene sequences revealed genetic variation among the examined isolates, with the greatest similarities belonging from isolates of the same owner/system. Two major lineages were observed; A and B, where lineage B had the majority of the isolates examined and all of the ones from MN. The location of isolation within the pig did not correlate with the dendrogram, however, in lineage A the majority of isolates were cultured from pleura. Isolates belonging to the same system clustered together, and in some instances isolates from different geographic location, but same system were identical. In contrast, three isolates from different systems, states and lesion type had a 100% sequence similarity. Both of these cases suggest a common source of pigs.

In summary, we have described an epidemiological tool for *M. hyorhinitis* typing. This tool will allow to further study the epidemiology and dynamics of infection. Moreover, it will be extremely useful for veterinarians and producers to understand disease outbreaks, to select isolates for vaccine production, and to perform epidemiological studies on the potential origin of a specific isolate. Therefore, the U.S. swine industry will be better positioned to control a pathogen that is responsible for an important part of the mortality observed in the nursery.

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Scientific Abstract:

M. hyorhinitis associated disease has been one of the main concerns of the U.S pork industry. It appears that differences in virulence of the infecting *M. hyorhinitis* strain, the host immune response, and concomitant infections may play a role on disease manifestation. There are currently no genotyping tools available for the characterization *M. hyorhinitis* isolates circulating amongst swine populations. The molecular typing of *M. hyorhinitis* would aid in better understanding transmission routes, in assessing sources of infection and also in evaluating interventions such as vaccination and use of antibiotics. The objective of this study was **to develop and validate a multi-locus sequence typing (MLST) protocol for the characterization of *M. hyorhinitis* field isolates**. Thirty-nine *M. hyorhinitis* field isolates together with one reference ATCC strain were utilized. The genome sequences of four *M. hyorhinitis* isolates were utilized to identify potential target genes. Primers were designed with MEGA 5. PCR was carried out and agarose gel electrophoresis was performed on the amplified products. PCR products were bidirectionally sequenced by standard Sanger sequencing. Quality of the

generated sequencing data was evaluated and sequences were aligned utilizing ClustalW and trimmed to equal sizes. Phylogenetic analysis was carried out using MEGA 5.2.1. A total of 25 genes were evaluated as potential target genes. Genes were discarded when the sequence of all 4 genomes were identical, when primers could not be designed due to high variability of the sequences, when no PCR amplification product was obtained or had a poor reproducibility. Finally, a total of 5 target genes were included in the MLST protocol: *ung*, *pdhB*, *mtlD*, *p3*, and *p95*. Within each gene the percent informative sites ranged from 0.5% to 20%. The number of alleles per gene varied from 3-11, giving rise to 27 sequence types (STs) within the 39 isolates. Two major lineages were observed. The concatenated tree showed clustering of isolates by system.

Introduction:

In recent years *Mycoplasma hyorhinis* has been recognized as an important cause of mortality in nursery pigs.¹ This pathogen is usually found in the upper respiratory tract of colonized pigs, more specifically in nasal secretions and in the oropharyngeal surface. Piglets become colonized by contact with the sows and it is transmitted through nose-to-nose contact among pigs afterwards.² *M. hyorhinis* causes polyserositis in 3-10 week old pigs, where animals show mainly fever, dyspnea, reluctance to move and unthriftiness. Infection in finishing stage pigs is usually characterized by arthritis; nonetheless, most *M. hyorhinis* infections are generally subclinical.³ Other clinical presentations including rhinitis, pneumonia, otitis and conjunctivitis have been reported for this organism. However, the role of *M. hyorhinis* in these disease presentations is unclear.

During the last years we have identified *Mycoplasma hyorhinis* as the main cause of polyserositis in many cases. In fact, 55% of polyserositis and 12% of arthritis cases received at the Minnesota Veterinary Diagnostic Laboratory tested positive for this pathogen by PCR.⁴ Although this pathogen was first described in 1955, very little research has been generated regarding the ecology and epidemiology of this organism, which is needed in order to design effective control and prevention protocols. Recently, our group had the opportunity to study the dynamics of *M. hyorhinis* infection in three conventional herds, through a field study funded by NPB in 2010.⁵ Interestingly, even though the three herds were very similar (same gilt and semen sources, same management and same veterinary care), two very different patterns of colonization and disease manifestation were observed. In two of these herds, most pigs became colonized with *M. hyorhinis* at the beginning of the nursery and *M. hyorhinis* was the main cause of nursery mortality. In contrast, pigs in the third herd did not become colonized until the last week of the nursery period and did not suffer from *M. hyorhinis* associated disease. One of the reasons why these similar herds showed such different *M. hyorhinis* infection dynamics could be the presence of different *M. hyorhinis* strains with different virulence. However, there are currently no tools available to investigate this hypothesis.

There are several pieces of information in the literature that suggest that there is heterogeneity in the *M. hyorhinis* species. Antigenic differences between different *M. hyorhinis* isolates have been shown by seroreactivity to specific antisera.⁶ This antigenic variation might be determined in part by the presence of a highly complex system of variable lipoprotein expression, which allows for great surface variation.⁷ A few experimental challenge studies have shown differences in virulence *in vivo*.^{8,9} Furthermore, the clinical presentation of disease caused by *M. hyorhinis* varies from herd to herd. While some herds are repeatedly affected by *M. hyorhinis*-associated arthritis, others tend to see mostly polyserositis (personal observations of PI). This different disease presentation also suggests that there are different strains with different tissue affinities.

In this project, we propose to develop genotyping techniques to differentiate *M. hyorhinis* strains. These techniques will be instrumental for scientists to understand the pathogenesis and epidemiology of this disease. At the same time, these tools will be extremely useful for veterinarians and producers to understand disease outbreaks, to select isolates for vaccine production, and to perform epidemiological studies on the potential origin of a specific isolate. After a thorough review of the bacterial genotyping methods available, Multiple-Locus Variable-Number Tandem- Repeats (MLVA) and Multiple-Locus Sequence Typing (MLST) were

chosen as the most suitable typing tools for this project. These are considered rapid and inexpensive techniques that identify phylogenetic relationships amongst diverse organisms.^{10,11}

Objectives:

1. To develop and validate the multiple locus variable number of tandem repeats analysis (MLVA) for the characterization of *M. hyorhina*.
2. To develop and validate the multi-locus sequence typing (MLST) technique for the characterization of *M. hyorhina*.
3. To identify genetic clusters of *M. hyorhina* field isolates from pigs with and without disease by multiple locus variable number of tandem repeats analysis (MLVA) and multiple locus sequence typing (MLST).
4. To compare the discriminatory power of multi-locus variable number of tandem repeats analysis (MLVA) and multiple locus sequence typing (MLST) on *M. hyorhina* field isolates.

Materials & Methods:

Bacterial isolates, media and DNA extraction

Thirty-nine *M. hyorhina* isolates from pigs with polyserositis and a clinical history suggestive of *M. hyorhina* infection (fever, depression, lameness, dyspnea), together with one reference ATCC strain (17981D) were employed in this study. Isolates were obtained from cases submitted to the UMN Veterinary Diagnostic Laboratory in 2010 and 2011, originated from 18 systems in 11 states and three pig sites; nursery, finishing and gilt acclimation units. Isolates were cultured from different regions within the pig; pleura, pericardium, joint, peritoneum, bronchus, nasal cavity and lung. Isolates were stored at -80 °C and grown in 3 mL aliquots of Modified Hayflick's medium for 7 to 14 days. Purified, genomic DNA was obtained using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

MLVA assay

The selection of the VNTR loci was carried out using the Tandem Repeat Finder software (www.tandem.bu.edu/trf/trf.html) and Microorganisms Tandem Repeats Database (<http://minisatellites.u-psud.fr/GPMS/>) on the existing genome sequence of *M. hyorhina* strain (HUB-1 NC_014448). The selection criteria for the VNTR loci selection was a size of 21-base pairs or larger, at least 2 copies per unit and repeated at least twice in the genome. Once the VNTR sequences were chosen, conserved primers were designed in the flanking region off the tandem repeat using PrimerQuest® (IDT, Coralville, USA) program software. The average amplification product of each gene was targeted to be around 500 base pairs.

Gene selection and primer design for MLST

The completed genome sequences of four *M. hyorhina* isolates available in Genbank were utilized to identify potential target genes. Identification of variable regions within the *M. hyorhina* species was accomplished through a multiple alignment of all 4 *M. hyorhina* genomes in Muave or the analysis of potential gene targets in MEGA 5.2.2. Primers were designed using PrimerQuest® (IDT, Coralville, USA) program and MEGA 5.2.1 (version 5; www.megasoftware.net). Primers targeted different housekeeping and surface protein encoding genes dispersed throughout the *M. hyorhina* genome. Target genes were PCR amplified using an initial denaturation step at 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, 43.5 °C for 1 min 30 sec. Agarose gel electrophoresis was performed on the amplified products and bands were observed under ultraviolet light in the presence of ethidium bromide. PCR

products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and bi-directionally sequenced by standard Sanger sequencing on an ABI 3730xl genetic analyzer (Applied Biosystems).

MLST data analysis

Quality of the generated sequencing data was evaluated and sequences were aligned utilizing ClustalW and trimmed to equal sizes. Evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analysis was carried out using MEGA 5.2. Minimum spanning tree analysis was generated using Bionumerics software V7.1. (Applied Math). The discriminatory power of the MLST assay was determined by calculating the Simpson's index of diversity for the MLST assay (Hunter *et al.*, 1988).

Results:

Objective 1:

MLVA assay:

Our first screen of the genome using Tandem Repeat Finder and Microorganisms Tandem Repeats Database revealed 4 previously described variant lipoprotein genes of *M. hyorhina* (*vlpC*, *vlpB*, *vlpD* and *vlpG*) that contain repeated motif (Figures 1-2). One VNTR candidate in the *vlpG* gene had a size of 30 base pairs, there were three copies per unit and the percent indels was 20%. Efforts to design and select the appropriate placement for the primers were unsuccessful. This was due, in part, to the low G+C content of *M. hyorhina*, which limits the area for primer assignment. In some instances where it was possible to design primers the amplicon size was above 600bp, which crosses the upper limit of reproducible sizing in most capillary electrophoresis platforms. Furthermore, the standardization of a published MLVA assay for *M. hyopneumoniae* in our group proved to be challenging. Consequently, it was decided to invest the resources and efforts to the promising MLST assay.

Objectives 2-4:

Gene selection

A total of 25 genes were evaluated as potential target genes. These included housekeeping genes: *pgmB*, *fusA*, *gyrB*, *lepA*, *metS*, *gltX*, *dnaA* and *pdhB*, surface proteins genes: *p3*, *p95*, *p37*, hexosephosphate transport protein (*hexo*), ribonucleoside-diphosphate reductase (*nrdF*), intracellular protein genes: uracil-DNA glycosylase (*ung*), mannitol-1-phosphate 5-dehydrogenase (*mtlD*), lipoprotein Signal peptidase II (*lspA*) and variable lipoproteins (*vlp*) *A,B,C,D,E,F* and *G*. The majority of the housekeeping genes and a few surface protein genes were found to be identical in all four annotated genomes, therefore were not included for further testing. On the contrary, the gene sequences for *vlpF* and *B* genes were found to be highly divergent among the four available genomes, with no conserved flanking regions hindering the possibility of primer placement. Primers for the remaining *vlp* genes were designed, however, an amplicon was not obtained for *vlpA*, *C*, *D* and *E* genes when testing 6 of the 39 available *M. hyorhina* isolates. Although an amplicon was generated for the *vlpG* gene, the generated product had low quality and was not reproducible. A total of 7 target genes were included in the MLST protocol: *ung*, *pdhB*, *mtlD*, *hexo*, *p3*, *p95* and *p37*. These genes produced the expected amplicons after PCR and yielded consistent high quality results after sequencing. These genes were distributed throughout the *M. hyorhina* genome (Figure 3).

MLST analysis

Variation within each gene ranged from 0.5 to 20%. The number of alleles per gene varied from 3-11, giving rise to 27 sequence types (STs) within the 39 isolates (Table 1). The discriminatory power of the technique was not affected after the removal of three genes; *p37*, *cls* and *hex*. In contrast, removal of any of the remaining 5 genes resulted in a lower number of STs and therefore in a decrease in the ability to differentiate strains. It appears that it might be sufficient to target only the remaining 5 genes. The dendrogram constructed based upon concatenated gene sequences (~ 3000bp) revealed genetic variation among the examined isolates (Figure 4). Two major lineages were observed; A and B. Nucleotide variation between lineages was approximately 3%. Within lineages variation ranged from 0 to 0.3%. Lineage B contained 26/39 isolates and all of the ones originating from MN. Lineage A contained 12/39. The majority of isolates in lineage A were cultured from pleura. Within lineage A, reference strain HUB-1, isolated in China, did not cluster with any of the remaining isolates within that lineage. No evident correlation was observed with respects to the age of the pig, lesion type, year or state. However, clustering by system was observed in nearly all cases. Isolates from different geographic location, but same system shared 100% sequence similarity. In contrast, three isolates from different systems, states and lesion type were also identical. Both of these cases suggest a common source of pigs. Minimum spanning tree analysis showed the distribution of the 27 STs into six clonal complexes (CC), with CC1 encompassing more isolates than any other CC (Figure 5). Simpson's index of diversity was calculated to be 0.978, which is the average probability that the typing scheme will assign different types to two unrelated isolates.

Discussion:

We have designed and evaluated an MLST typing scheme for *M. hyorhinis*. We have identified genetic variation within the *M. hyorhinis* species in several genes.. Using this MLST typing tool, all isolates belonging to the same production system clustered together, proving the usefulness of the assay. Currently swine producers and veterinarians are faced with the challenge of controlling *M. hyorhinis* disease in affected post-weaning pigs. The use of a genotyping tool will result in a better understanding of the diversity of *M. hyorhinis* field isolates circulating in U.S swine herds. This tool will allow studying the epidemiology and dynamics of infection for this pathogen. Furthermore, this tool will be extremely useful for veterinarians and producers to understand disease outbreaks, to select isolates for vaccine production, and to perform epidemiological studies on the potential origin of a specific isolate. Therefore, the U.S. swine industry will be better positioned to control a pathogen that is responsible for an important part of the mortality observed in the nursery.

Tables and figures:

Figure 1- Output obtained from the Tandem Repeat software after screening the *M. hyorhinis* HUB-1 complete genome. Arrows indicate two areas with tandem repeats that fit the selection criteria. First arrow from top to bottom indicates tandem repeat found in gene *vlpD* and *vlpG*, respectively.

253641--253688	21	2.2	22	85	3	62	20	14	4	60	1.51
270594--270628	18	1.9	18	88	11	54	34	20	8	37	1.83
Indices	Period Size	Copy Number	Consensus Size	Percent Matches	Percent Indels	Score	A	C	G	T	Entropy (0-2)
291445--291483	20	1.9	21	84	5	53	61	30	0	7	1.24
294198--294241	18	2.4	18	81	7	54	54	18	9	18	1.69
323567--323621	18	2.8	19	86	8	67	52	3	0	43	1.18
339043--339076	13	2.6	13	90	9	52	0	0	14	85	0.60
352097--352138	21	2.0	21	86	9	59	50	19	9	21	1.75
352131--352188	20	2.9	21	70	12	59	63	5	10	20	1.44
353309--353343	15	2.3	15	100	0	70	48	5	14	31	1.67
364937--364966	15	2.0	15	93	0	51	40	16	13	30	1.87
384038--384077	9	4.3	9	87	12	55	12	0	12	75	1.06
384038--384077	19	2.2	19	86	13	57	12	0	12	75	1.06
400084--400129	21	2.1	23	84	8	60	54	0	15	30	1.41
433019--433066	20	2.5	20	77	9	57	62	0	0	37	0.95
433666--433748	45	1.9	42	85	7	112	28	19	27	24	1.98
433841--434008	36	4.7	36	95	0	300	45	19	13	21	1.84
434579--434622	18	2.4	18	76	0	52	29	20	27	22	1.99
434665--434933	117	2.3	117	93	0	466	44	30	5	18	1.74
434656--434948	39	7.5	39	90	0	406	44	29	7	18	1.76
437588--438142	66	7.9	66	90	4	489	35	22	21	20	1.96
437660--437932	78	3.5	78	100	0	546	38	21	21	18	1.94
437896--437995	30	3.1	30	64	20	76	36	23	19	22	1.95
438948--439592	39	16.5	39	99	0	1263	54	22	15	8	1.67
441655--441711	24	2.4	24	81	0	69	33	21	26	19	1.97
Indices	Period Size	Copy Number	Consensus Size	Percent Matches	Percent Indels	Score	A	C	G	T	Entropy (0-2)



Figure 2- Output obtained from the Microorganisms Tandem Repeats Database after screening the *M. hyorhinis* HUB-1 complete genome. Arrows indicate two areas with repeated motif that fit the selection criteria. First arrow from top to bottom indicates tandem repeat found in gene *vlpG*, *vlpB* and *vlpC*, respectively.

Characteristics of tandem repeats according to following criteria :

1. Total length between 50 and 1000
2. Unit length between 2 and 200
3. Copy number between 2 and 100
4. Percent matches between 80 and 100

Physical position (kb)	Contig name	Unit length	Copy number	Total length	Percent matches	%C	%G	%T	%A	GC bias
48	Mycoplasma_hyorhinis_HUB_1	30	2.0	58	80%	10%	3%	64%	22%	0.53
58	Mycoplasma_hyorhinis_HUB_1	12	10.0	118	82%	8%	5%	47%	38%	0.23
66	Mycoplasma_hyorhinis_HUB_1	29	2.0	57	80%	10%	5%	56%	27%	0.33
167	Mycoplasma_hyorhinis_HUB_1	24	2.2	50	80%	7%	9%	21%	60%	0.12
208	Mycoplasma_hyorhinis_HUB_1	28	2.1	53	80%	7%	5%	59%	27%	0.16
337	Mycoplasma_hyorhinis_HUB_1	23	2.1	50	82%	5%	7%	39%	47%	0.16
433	Mycoplasma_hyorhinis_HUB_1	36	4.7	167	95%	19%	13%	21%	45%	0.18
434	Mycoplasma_hyorhinis_HUB_1	39	7.6	296	90%	29%	7%	18%	44%	0.61
437	Mycoplasma_hyorhinis_HUB_1	66	8.0	561	90%	22%	21%	20%	35%	0.02
439	Mycoplasma_hyorhinis_HUB_1	39	16.5	644	99%	22%	15%	8%	54%	0.18
441	Mycoplasma_hyorhinis_HUB_1	36	13.1	474	95%	17%	25%	17%	40%	0.19
442	Mycoplasma_hyorhinis_HUB_1	36	17.3	623	97%	27%	15%	16%	40%	0.28
472	Mycoplasma_hyorhinis_HUB_1	28	2.3	61	82%	6%	8%	53%	32%	0.14
815	Mycoplasma_hyorhinis_HUB_1	36	2.8	99	90%	14%	34%	34%	18%	0.41

14 sequences correspond to criteria



Figure 3- Circular representation of *M. hyorhina* HUB-1 chromosome and location of targeted genes. The outer circle depicts scale in base pairs. Circles 2-3 indicate coding regions. Circle 4 depicts G+C content relative to the genome average of 26% (purple=below average, green=above average). The position of the target genes is indicated by colored bars transversal to the circles that indicate coding regions.

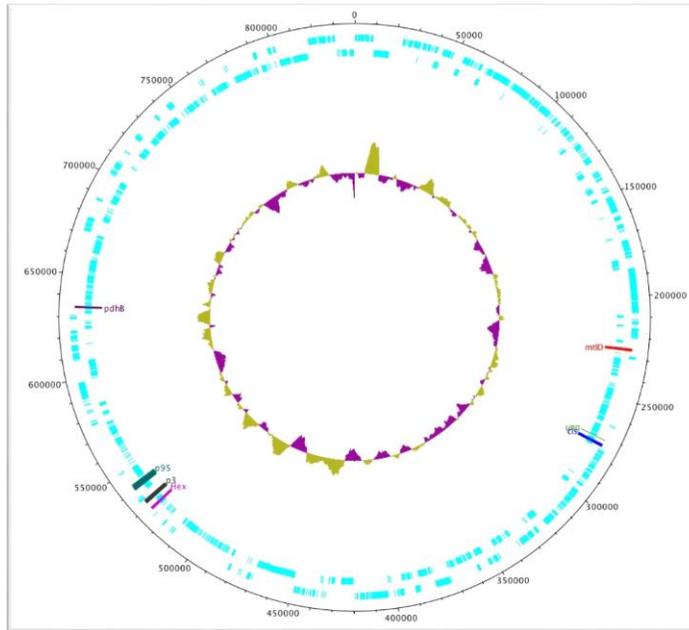
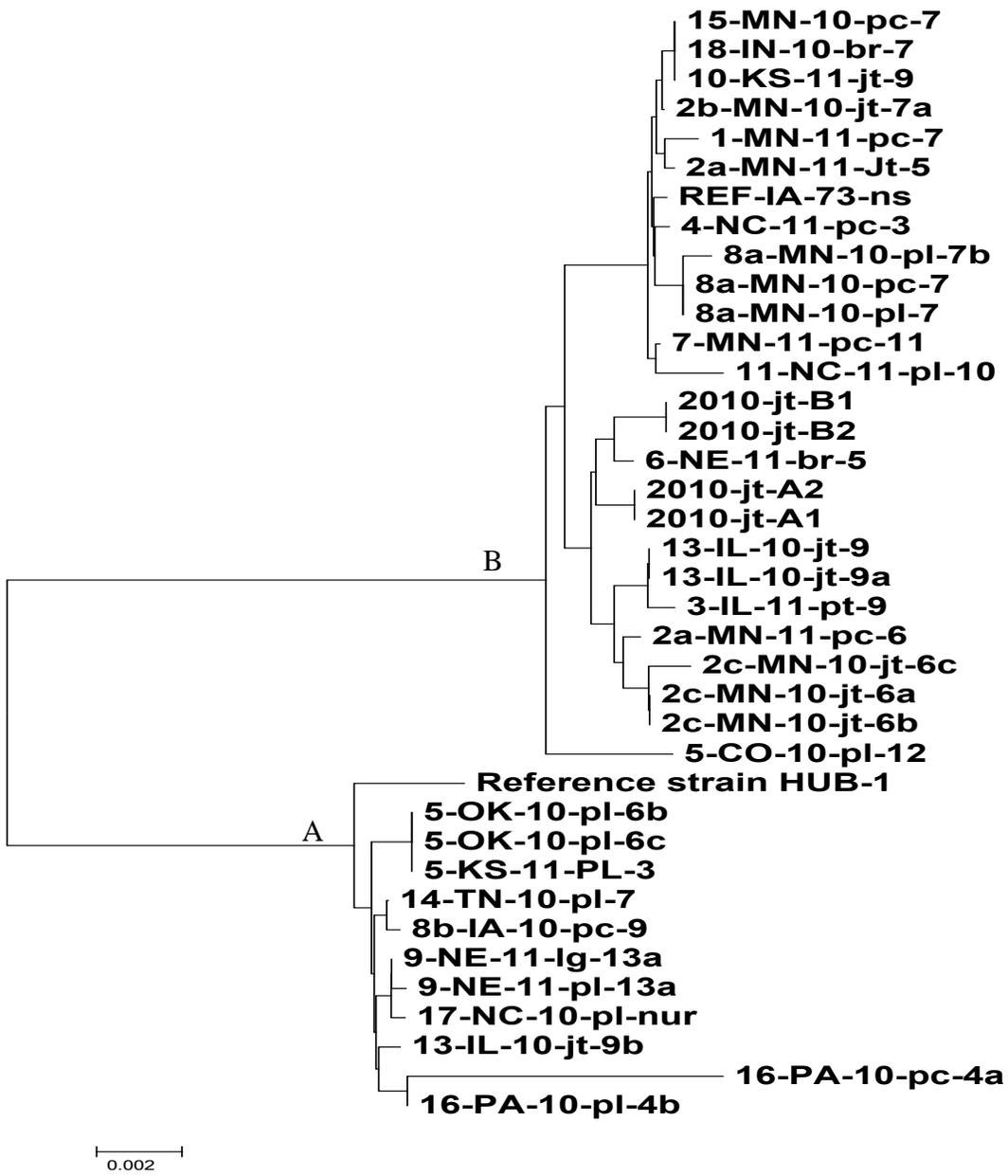


Table 1- Characteristics of regions used for multilocus sequence analysis and number of alleles

Target gene	Name	Size (kb)	Trimmed length	Variable sites	Informative sites	Percent informative sites	No. of alleles
<i>pdhB</i>	Pyruvate dehydrogenase-E beta-1 subunit	1.28	389	4	2	0.5	5
<i>hex</i>	Hexosephosphate transport protein	4.12	334	9	5	1.5	9
<i>p95</i>	Outer membrane protein p95	2.84	173	2	2	1.2	2
<i>cls</i>	Cardiolipin synthetase	1.88	381	2	1	0.3	4
<i>p3</i>	Outer membrane protein p3	0.87	404	5	4	0.9	3
<i>mtlD</i>	Mannitol-1-phosphate 5-dehydrogenase	1.43	378	86	79	20	11
<i>ung</i>	Uracil-dna glycosylase	2.02	562	3	1	0.2	5

Figures 4- Inferred relationships between 39 *M. hyorhinis* isolates using concatenated sequences for genes: *pdhB*, *p95*, *p3*, *mtlD* and *ung*. Identification of each isolate is based on a key (owner number-state-year-lesion type-age of pig). Letters above main branches represent lineages. Data was analyzed in MEGA5 2.2 using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. A total of 3,014 positions were employed in the final dataset. In total 27 sequence types (STs) were identified from the 39 isolates examined.



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