

## Genetic Diversity of *Mycoplasma hyosynoviae* Field Isolates in Thailand

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### Abstract

Pulsed-field gel electrophoresis (PFGE) and Random amplified polymorphic DNA (RAPD) analyses were performed to compare techniques and to investigate genetic diversity as an epidemiological data of Thai *M. hyosynoviae* isolates. A total of 42 isolates including a type strain S16 was typable and consisted of 39 different patterns by RAPD technique, whereas 37 isolates (97%) were typable and consisted of 22 different patterns by PFGE technique. Based on PFGE patterns, multiple clones of *M. hyosynoviae* were generally present in pig farms, whereas high genetic heterogeneity of *M. hyosynoviae* among the pig farms was shown. No identical PFGE pattern between the pig farms was found except two farms that were located in the same province. This finding might indicate the distribution of the organism from the same source. Monitoring the genetic diversity of *M. hyosynoviae* strains using PFGE analysis should be useful to elucidate the epidemiology of *M. hyosynoviae* infections in Thailand.

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**Keywords:** genetic diversity, *Mycoplasma hyosynoviae*, PFGE, RAPD, Thailand

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## บทคัดย่อ

### ความหลากหลายทางพันธุกรรมของเชื้อ มัยโคพลาสมา ไฮโอซินโนวีแ่ ที่เพาะได้ในประเทศไทย

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เมื่อนำเทคนิค PFGE และ RAPD มาเปรียบเทียบความสามารถในการแยกและศึกษาความแตกต่างทางพันธุกรรมเพื่อเป็นข้อมูลทางระบาดวิทยาของเชื้อมัยโคพลาสมา ไฮโอซินโนวีแ่ที่เพาะได้ในประเทศไทยจำนวน 41 สายพันธุ์และเชื้อสายพันธุ์อ้างอิง S16 พบว่า วิธี RAPD สามารถแยกความแตกต่างของเชื้อทั้ง 42 สายพันธุ์ออกได้ทั้งสิ้น 39 รูปแบบ ในขณะที่มีเชื้อจำนวน 37 สายพันธุ์ หรือคิดเป็นร้อยละ 97 ที่เกิดแถบดีเอ็นเอโดยวิธี PFGE และแยกความแตกต่างออกได้เป็น 22 รูปแบบ การวิเคราะห์ผลของวิธี PFGE พบการอยู่เป็นกลุ่มของเชื้อที่มาจากสายพันธุ์ต้นกำเนิดเดียวกันในแต่ละฟาร์ม ในขณะที่เชื้อที่เพาะได้จากต่างฟาร์มกันมีความหลากหลายทางพันธุกรรมค่อนข้างสูง โดยแทบไม่พบว่ามีรูปแบบ PFGE แบบเดียวกันในเชื้อที่มาจากต่างฟาร์มกัน ยกเว้นมี 2 ฟาร์มที่ตั้งอยู่ในจังหวัดเดียวกันที่พบเชื้อที่มีรูปแบบเดียวกัน ซึ่งให้นิพจน์ว่านั่นคือการกระจายของเชื้อที่มาจากแหล่งหรือต้นกำเนิดเดียวกัน การติดตามลักษณะความหลากหลายทางพันธุกรรมของเชื้อมัยโคพลาสมา ไฮโอซินโนวีแ่โดยวิธี PFGE จะเป็นประโยชน์ในการทำให้เกิดความชัดเจนด้านระบาดวิทยาของการติดเชื้อมัยโคพลาสมา ไฮโอซินโนวีแ่ในประเทศไทย

**คำสำคัญ:** ความหลากหลายทางพันธุกรรม มัยโคพลาสมา ไฮโอซินโนวีแ่ ประเทศไทย

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## Introduction

*Mycoplasma hyosynoviae* is one of the porcine mycoplasma pathogens distributed worldwide. It commonly inhabits the upper respiratory airways and tonsils of adult pigs. Pigs are occasionally infected with and developed pneumonic lesion, non-suppurative arthritis and lameness. However, the development of pneumonia and/or arthritis might depend on many factors including variation of virulent factors and antigenicity of different strains (Hagedorn-Olsen et al., 1999). Nowadays, arthritis caused by *M. hyosynoviae* becomes increasing problem in many countries (Nielsen et al., 2001; Assuncao et al., 2005; Dahlia et al., 2009). Recently, the occurrence of three porcine mycoplasmas including the first demonstration of *M. hyosynoviae* infection in Thailand identified by isolation technique and PCR was reported (Makhanon et al., 2012). The semi-nested PCR was helpful in screening the presence of *M. hyosynoviae* in the farms. Consequently, the organisms could be isolated from the pigs in various farms (Thongkamkoon et al., 2012). Genomic characterization of *M. hyosynoviae* strains using various molecular typing methods is helpful in

revealing the intraspecies genomic variations and facilitating epidemiological studies. Selection of molecular typing technique for mycoplasma species might depend on genetic basis of the species, typability and discriminatory power of the technique as well as laboratory facility. Amplified fragment length polymorphism (AFLP) has been used for typing various mycoplasma species of human and animal origin and *M. hyosynoviae* showed highly genetic difference among isolates which were observed by AFLP and pulsed-field gel electrophoresis (PFGE) analysis techniques (Kokotovic et al., 1999). These two techniques yielded the comparable results for differentiation of *M. hyosynoviae* strains obtained from different geographical locations. The identical patterns were detected only for the strains obtained from the same country suggesting the ability of the methods in monitoring the epidemiological relatedness of the strains. Moreover, the discriminatory power for differentiation of *M. hyosynoviae* isolates of AFLP was equal to PFGE analysis (Kokotovic et al., 2002a). Random amplified polymorphic DNA (RAPD) analysis was developed and found to be useful to investigate the epidemiologically related strains of avian mycoplasmas. For *M. synoviae*, RAPD has been

used for differentiation of subspecies level (Fan et al., 1995). However, comparison between RAPD and PFGE was evaluated and found that typability and discriminatory power of RAPD were still greater than PFGE (Marois et al., 2001). For a genetically homogeneous species, *M. pneumoniae*, PFGE and RAPD had a little advantage over RFLP analysis of the P1 gene (Cousin-Allery et al., 2000). A high diversity was observed for the strains of *M. bovis* by RAPD technique (McAuliffe et al., 2004). Although it seemed to have lower discriminatory power for differentiation of *M. hyopneumoniae* strains (Stakborg et al., 2006), the discrimination power of RAPD to *M. hyosynoviae* was doubtful since it has never been used for intraspecies study of *M. hyosynoviae*.

The aim of the present study was to compare PFGE and RAPD techniques for molecular typing of *M. hyosynoviae* and to investigate the genetic diversity as an epidemiological data of Thai *M. hyosynoviae* isolates. The relationship between the genetic patterns and partial sequence of 23S rRNA gene of the isolates was also demonstrated.

### Materials and Methods

**Mycoplasma strains :** Forty one Thai isolates of *M. hyosynoviae* isolated from slaughtered pigs during 2008-2011 at National Institute of Animal Health, Thailand and kept at -80°C were used. Type strain S16 obtained from National Institute of Animal Health, Japan was also included. Of the Thai isolates, 18 were isolated from tonsils, synovial fluid and lung of pigs from 5 farms located in Chiang Mai (1), Buri Rum (2), Nakhon Sawan (1) and Nakhon Ratchasima (1) in 2008, 13 were isolated from tonsils of pigs from one farm located in Chon Buri in 2009 and 10 were isolated from lungs and tonsil of pigs from 3 farms located in Suphan Buri, Nakhon Ratchasima and Saraburi in 2011. Each of *M. hyosynoviae* isolates was propagated in 30 ml Hayflick's broth with arginine and mucin (Friis et al., 1991) and incubated in 37°C for 4 days. All Thai isolates were determined for an acquired resistance to macrolides and lincomycin by sequence analysis of domains II and V of their 23S rRNA gene in the previous study (unpublished data).

**Pulsed-field gel electrophoresis (PFGE) :** *M. hyosynoviae* cells were harvested from the broth culture by centrifugation at 10,000 xg for 30 min at 4°C (Himac CR22GII, Hitachi, Japan). The packed cells were washed three times in 2 ml washing buffer (50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, pH 7.2) and centrifuged at 13000 xg for 5 min at 4°C (Model 1920, Kubota, Japan). The cells were resuspended in 200 µl of normal saline. The suspension was mixed with an equal volume of 1.6% low melting agarose (Bio-Rad, USA) and loaded into a plug mould (Bio-Rad, USA) set at 4°C for 10 min. Then, the agarose plugs were left in 2 ml lysis buffer (10 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 1% N-lauryl sarcosine, 0.1 mg/ml proteinase K) at 50°C overnight. After incubation, the plugs were washed three times in DW for 15 min at 50°C, followed by washing in TE for 15 min at 50°C for two times. Prior to DNA digestion, the

plugs were equilibrated in 1x restriction enzyme buffer at 37°C for 10 min. Thirty units per plug of BssHII restriction enzyme (New England Biolabs, USA) was used for the digestion of genomic DNA of *M. hyosynoviae* at 37°C for 5 hours.

PFGE was performed with a CHEF-DR III Pulsed Field Electrophoresis System (Bio-Rad, USA). After digestion by restriction enzyme, the plugs were rinsed one time by 0.5x TBE buffer and loaded into 1.0% pulsed field certified agarose (Bio-Rad, USA). Electrophoresis was run at 140°C and 6V/cm from 0.5-8.5 sec switching times for 18 hours. After electrophoresis, the agarose gels were stained with 0.1 µg/ml ethidium bromide in DW, and destained in DW and DNA fragments were visualized and photographed under ultraviolet light in a gel documentation system (GelDoc-It, UVP, USA).

**Random amplified polymorphic DNA (RAPD):** One ml of each *M. hyosynoviae* culture was harvested at 10,000 xg for 10 min (Model 1920, Kubota, Japan). Then, the pellet was washed once with 1 ml PBS and mixed with Instagene matrix (Bio-Rad, USA) for DNA preparation following the manufacturer's instruction. RAPD was performed for all samples in one single PCR using Ready-To-Go RAPD analysis kit (GE Healthcare, Life Sciences, USA) following the manufacturer's instruction. Briefly, five µl of DNA template, 25 pmol of RAPD analysis primer 6-[5'-d(CCCGTCAGCA)-3'] and 15 µl of distilled water were added into a tube containing RAPD analysis bead. The contents were mixed gently by vortex. A sample was placed in a thermal cycler (Hybaid, Thermo electron, USA) using the following program: 1 cycle of 95°C for 5 min, 45 cycles of 95°C for 1 min, 36°C for 1 min and 72°C for 2 min. Amplification products were electrophoresed through 2% agarose gel containing 0.1 µg/ml ethidium bromide at 100 volts for 50 min. RAPD patterns were also visualized and photographed under ultraviolet light.

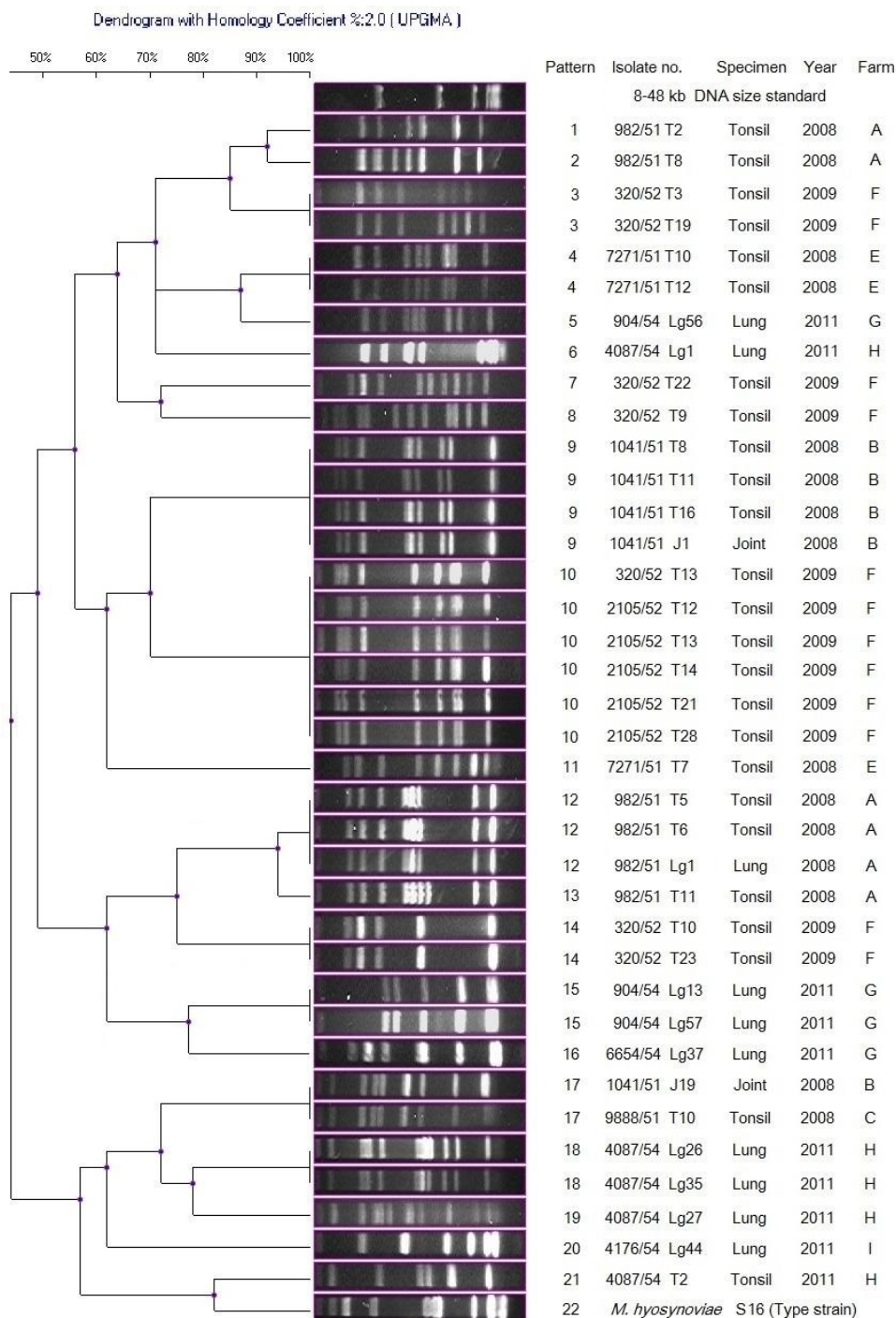
**Data analysis :** Dendrogram, based on the PFGE patterns, was constructed using BIO-PROFIL Bio-1D++ v11.11 software (Vil-Lourmat, Germany). Similar matrix between PFGE profiles was calculated using Dice similarity coefficient. Cluster analysis was performed with unweighted pair group method using average linkages (UPGMA). Isolates that had 100% similarity were considered to be the same strain and isolates that had more than 90% similarity were considered to be genetically related isolates and to be derived from a common parent. The dendrogram, based on the RAPD patterns, was also constructed with the same software.

### Results

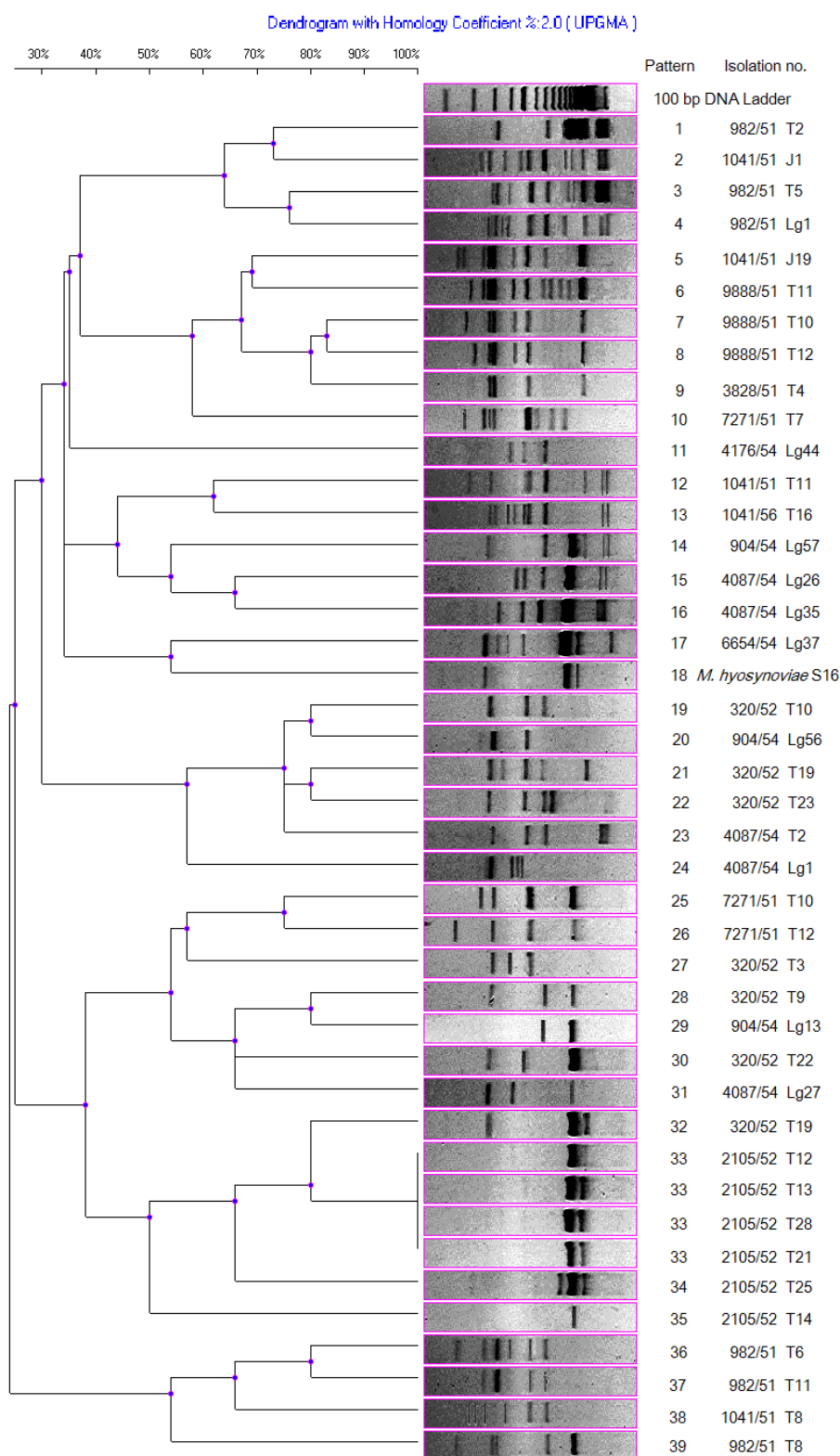
**PFGE :** Of 41 Thai *M. hyosynoviae* isolates, 37 isolates were typable by PFGE technique whereas 4 isolates which consisted of 2 isolates from farm B and one isolate each from farm D and F did not yield well-separated bands. The banding patterns of typable strains consisted of 6-12 fragments in the size range of 2-48 kb. Twenty two different patterns were detected among 37 Thai *M. hyosynoviae* isolates and the type strain S16 (Fig 1). Six isolates obtained from farm A

differentiated into 4 PFGE patterns including patterns 1, 2, 12 and 13. Similarity between pattern 1 and 2 was more than 90%. The same result was also shown between pattern 12 and 13. Of the 5 isolates obtained from farm B, 4 isolates had 100% similarity of PFGE pattern 9 whereas the other isolate showed PFGE pattern 17 which had 100% similarity to the isolate obtained from farm C. Three isolates obtained from farm E showed PFGE patterns 4 and 11. Eight isolates obtained from the first fattening pig herd in farm F showed PFGE patterns 3, 7, 8, 10 and 14, whereas 5 isolates obtained from another fattening pig herd that

was raised two months later in the same farm showed the same PFGE pattern 10. Three isolates obtained from farm G showed PFGE patterns 5 and 15. Isolate obtained from different fattening pig herd that was raised four months later in the same farm showed PFGE pattern 16 which had 75% similarity to pattern 15. The isolate obtained from farm I showed the PFGE pattern 20. Five isolates obtained from farm H showed PFGE patterns 6, 18, 19 and 21. The *M. hyosynoviae* type strain S16 showed PFGE pattern 22, which was distinguishable from the local isolates.



**Figure 1** Dendrogram of PFGE fragments of 37 Thai *M. hyosynoviae* isolates and type strain S16. Cluster analysis was performed with UPGMA using Dice similarity coefficient and 2% interval of confidence for band matching.



**Figure 2** Dendrogram of RAPD profiles of 41 Thai *M. hyosynoviae* isolates and type strain S16. Cluster analysis was performed with UPGMA using Dice similarity coefficient and 2% interval of confidence for band matching.

In this study, the typability of PFGE to 41 Thai *M. hyosynoviae* isolates was 91%. PFGE technique had a high reproducibility with the identical banding pattern obtained for replicate samples, even when the DNA extraction and electrophoresis were performed at different time.

**RAPD** : Five randomly selected Thai *M. hyosynoviae* isolates and the type strain S16 were typable by RAPD technique using 6 RAPD analysis primers and RAPD beads, however different discriminatory powers were found among the primers (result not shown). RAPD analysis primer 6 was selected due to the highest discriminatory power. The reproducibility was stable

only when RAPD was performed using the RAPD beads since the results obtained from different pre-prepared commercial mastermix showed different fingerprints (result not shown). The banding patterns consisted of 1-12 fragments in the size range of 200-1500 bp. Thirty nine RAPD patterns were detected among 41 Thai *M. hyosynoviae* isolates and the type strain S16 (Fig 2). All isolates obtained from the different farms showed different RAPD patterns and almost all isolates obtained from the same farms showed different RAPD patterns. The exception was 4 isolates obtained from farm F showing 100% similarity pattern 33 which was in agreement with the result obtained from PFGE analysis. The three isolates from farm C, of which 2 isolates could not be typed by PFGE, showed their RAPD patterns (patterns 6, 7, 8) within the group with 67% similarity (Fig 2). The RAPD patterns 7 and 8 which had about 83% similarity belonged to the isolates obtained from the same farm whereas RAPD 3 and 4 which had about 77% similarity belonged to the isolates showing the same PFGE profile.

*Relationship between genetic profiles and sequences of domains II and V of 23S rRNA* : An acquired G745A transition in domain II was found in Thai *M. hyosynoviae* isolates including all 6 isolates from farm A: 4 isolates from farm G, of which 2 isolates showed an identical PFGE pattern 15 and 1 isolate showed the related PFGE pattern 16; 3 isolates from farm H, of which 2 isolates showed an identical PFGE pattern 18. In addition, 2 isolates from farm H showing an identical PFGE pattern 3 obtained an acquired C739T transition in domain II.

### Discussion

As revealed by PFGE, the clonal appearance was found in farm A, B, E, F, G and H. The same clone found in different organs of pigs including pattern 9 in farm B and pattern 12 in farm A might indicate the invasion ability of *M. hyosynoviae* strains from tonsil to either joint or lung of the pig. The 92% similarity between patterns 1 and 2, and the 95% similarity between patterns 12 and 13 in farm A demonstrated the alteration in genetic composition of one clone during its persistence in the herd. Diversity of Danish *M. hyosynoviae* strains, using AFLP analysis, consisted of 13 identical patterns or clonal appearance for 2 to 12 strains obtained from the same farms and the strains obtained from the same herds had either 1 or 4 different clonal lines (Kokotovic et al., 2002<sup>b</sup>). Similar result was found in this study since 2 to 5 clonal lines were also found in farms A, B, E, F, G and H supporting the evidence of highly genetic heterogeneity of *M. hyosynoviae*. Sharing of identical pattern between two strains obtained the different geographic locations in Denmark was observed once in the previous study (Kokotovic et al., 2002<sup>b</sup>). In contrast, we found only a sharing of the same clonal of pattern 17 obtained from different farms (farm B and farm C) within the same province. Thus, they might get the piglets from the same source. However, sharing the identical pattern of *M. hyosynoviae* obtained from different geographic location in Thailand might be observed following our

investigation in the future.

In this study, RAPD revealed almost 100% of genetically heterogeneous *M. hyosynoviae*. Although the result could be complicated due to inconsistent band intensities, this finding might support that a high rate of changes in *M. hyosynoviae* genome was possible (Kokotovic et al., 2002<sup>b</sup>). In contrast, many genetically heterogeneous *M. hyopneumoniae* isolates and a few single clones distributed among the pig herds. A single clonal line was common inside one herd (Stakenborg et al., 2005). Despite the fact that only specific clones were responsible for the outbreak among the farms in close geographic location (Mayor et al., 2007), strong evidence of multiple clones of *M. hyopneumoniae* circulating in a single pig or pigs within one herd was observed by RAPD analysis (Nathues et al., 2011). Those results might indicate that *M. hyopneumoniae* is a lower genetically heterogeneous species compared to *M. hyosynoviae*, resulting in a chance to have identical RAPD profiles of *M. hyopneumoniae* among pig population.

Although RAPD seemed to have typability and discriminatory potential greater than PFGE in this study, we preferred using PFGE to reveal genetic diversity of *M. hyosynoviae* in Thailand. The most important drawback of RAPD was the limited reproducibility of the technique although all standard reagents and materials were used. Number and intensity of the bands might vary due to more or less DNA template, resulting in analysis problems. In addition, RAPD result was not related to the farms or origins of the isolates, whereas PFGE results showed a good correlation of its patterns and the origins of the isolates. Interestingly, the relationship between the alteration of 23S rRNA gene and PFGE pattern of *M. hyosynoviae* isolates within farms A, G and H was demonstrated, which supported the reliability of PFGE results and obviously confirmed that PFGE would be a suitable and useful tool for epidemiological study of *M. hyosynoviae*.

In conclusion, the typability of RAPD and PFGE for Thai *M. hyosynoviae* isolates in this study was 100% and 91%, respectively. RAPD profiles showed very high genetic heterogeneity of *M. hyosynoviae* isolates inside the herd and among the herds, whereas PFGE profiles could be grouped and revealed a single or multiple clonal lines of *M. hyosynoviae* inside the herd and between the herds within the same province. Although *M. hyosynoviae* is considered a highly genetic heterogeneity, a longitudinal observation for their subspecies PFGE patterns among pig population throughout the country will be useful to elucidate the epidemiology of *M. hyosynoviae* infections in Thailand.

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