strains that have evolved over different time spans (Chiou et al., 2009; Keim et al., 2004). In the last decade, many multilocus VNTR analysis (MLVA) methods have been developed for the fine typing and phylogenetic analysis of bacterial pathogens (Liang et al., 2007; Lindstedt, 2005; Wang et al., 2009). In practice, an MLVA protocol based on a small set of highly mutable or variable VNTRs can have extremely high resolving power for discriminating closely related isolates, whereas a larger set of VNTRs is more favorable to obtain a clearer separation of distinct phylogenetic groups.

For *S.* Typhimurium, an MLVA5 protocol, based on five VNTRs (STTR3, STTR5, STTR6, STTR9, and STTR10), has been developed and widely used in many European laboratories (Lindstedt et al., 2004). Another MLVA protocol based on a set of seven VNTRs has been used in the laboratory of US Centers for Disease Control. Because five *S.* Typhimurium genomic sequences are available, a large number of VNTRs can be explored in silico from the five genomes using a computer program, such as VNTRDB (Chang et al., 2007). In this study, we explored a large number of VNTRs from five *S.* Typhimurium genomes using the VNTRDB computer program. The VNTRs were further tested with panels of a large number of isolates to assess their allelic diversity, variability and stability, to compare the discriminatory power for PFGE and various MLVAs (based on various combinations of VNTRs) and to evaluate the usefulness of MLVA data in delineating phylogenetic structure among *S.* Typhimurium isolates.

2. Materials and methods

2.1. Bacterial strains

A total of 440 *S.* Typhimurium isolates were used in this study. Of the 440 isolates, 386 were selected from a collection of 2,840 *S.* Typhimurium isolates that had been collected from 44 collaborative hospitals across Taiwan from 2004 to 2008 and were characterized using PFGE in our laboratory. The other 54 were recovered from seven foodborne outbreaks occurring in 1998–2003. The 386 isolates were placed in two panels, P183 and P203. P183 consisted of 183 isolates randomly selected among the isolates in the *S.* Typhimurium databases with minor PFGE genotypes; these isolates were considered more genetically diverse. P203 consisted of 203 isolates randomly selected among isolates with eight prevalent PFGE genotypes; isolates sharing a common PFGE pattern were considered closely related. Forty diverse isolates within the P183, which shared a low level of PFGE similarity, were used in a preliminary test to evaluate the polymorphism of the VNTRs in silico explored from five sequenced *S.* Typhimurium genomes.

2.2. PFGE

Isolates were characterized using the PulseNet PFGE protocol for the subtyping of *Salmonella* (Ribot et al., 2006), except that 5 U instead 40 U of *Xba*I was used for the restriction digestion. PFGE images were digitally recorded in tiff file format using a Kodak EDAS290 System (Eastman Kodak Co, Rochester, NY, USA) for further analysis.

2.3. Exploring VNTR loci

The five genomic sequences of *S.* Typhimurium strains LT2 (accession no. NC_003197), DT104, DT2, SL1344, and D23580 (obtained from

http://www.sanger.ac.uk/Projects/*Salmonella*/) were used to explore VNTRs using the VNTRDB computer program (Chang et al., 2007). VNTRs found by the in silico searches were evaluated for their polymorphisms with a panel of 40 diverse isolates using PCR. To perform PCR reactions, primers for the loci were designed using the Primer3 program (http://frodo.wi.mit.edu/). PCR reactions were carried out in a GeneAmp PCR System 9600 (Applied BioSystems). For PCR amplification, crude bacterial DNA was prepared by the boiling method as described (Liang et al., 2007). Each 10-μl PCR mixture contained 1x PCR buffer, 3 mM $MgCl_2$, 0.2 μM of each primer, 200 μM of each deoxyribonucleotide, 1.0 U of the recombinant SuperNew *Taq* DNA polymerase (Jier Sheng Company, Taipei, Taiwan), and 1 μl of DNA template. The PCR reaction was performed with a denaturing step at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Loci with varied sizes of amplicons among the 40 isolates were chosen as the subjects for further characterization in a total of 440 isolates.

2.4. MLVA

The primers, dyes and multiplex PCR combinations for 16 VNTR loci are listed in Table 1. The forward primer for each primer set was labeled at its 5' end with an ABI-compatible dye, 6-FAM, NED, VIC or PET by Applied BioSystems (Foster City, CA, USA). Four multiplex PCR combinations were set for the analysis. PCR reactions were performed as described above except that dye-labeled primers and various primer concentrations were used (Table 1). Occasionally, no amplicon was detected for some loci in multiplex PCR reactions; in these cases, the loci were amplified individually. If no amplicon was detected by individual locus amplification, DNA of these isolates was prepared by a commercial kit (Geneaid, Taipei County, Taiwan) for amplification. The PCR products were analyzed by capillary electrophoresis on an ABI

Prism 3130 Genetic Analyzer with GeneScan 500 LIZ Size Standard (cat # 4322682; Applied BioSystems) as described previously (Liang et al., 2007).

2.5. Data analysis

PFGE images were analyzed using the fingerprint analysis software BioNumerics version 4.5 (Applied Maths; Kortrijk, Belgium). The PFGE pattern for each isolate was saved in a *Salmonella* DNA fingerprint database established by the Taiwan Centers for Disease Control (Taiwan CDC). The number of repeat units for each allele was converted from the size of amplicon and saved as "Character Type" in the BioNumerics *Salmonella* database. STTR3 is a composite locus with 27-bp and 33-bp repeats and could bear imperfect repeat(s), similar to STTR7, resulting from insertion or deletion in the repeat array region; alleles for these two loci were designated by the sizes of repeat array regions. The VNTR profiles were subjected to cluster analysis using the minimum spanning tree algorithm and categorical coefficient provided in the BioNumerics software. The creation of hypothetical types (missing links) was permitted in order to introduce hypothetical types, group minimum size was set at 2, and maximum neighbor distance was set at 3. To compare the discriminatory power of PFGE and various MLVAs that were based on various combinations of VNTRs, Simpson's diversity index (DI) and 95% confidence intervals (CI) were calculated according to the formulas as the described (Grundmann et al., 2001; Hunter, 1990). The polymorphism of each locus was represented by Nei's diversity index, calculated as $1-\sum(\text{allelic frequency})^2$. The Nei's diversity index for the panel of P203 was calculated by giving weights to the number of isolates for each of the eight PFGE types. The eight PFGE patterns for the P203 isolates were compared with those with phage typing data in the Danish database. A PFGE pattern that matched a Danish

strain was considered to bear the phage type of the Danish strain.

3. Results

3.1. Characterization of the VNTRs

A total of 39 VNTRs were identified in silico from five *S.* Typhimurium genomic sequences (Table 2). These loci have repeats of lengths ranging from 3 bp to 200 bp, 87% to 100% repeat sequence matches, and no indels. One of these, STTR10, is carried on the invasive plasmid (pSLT in strain LT2). Six loci (STTR3, STTR5, STTR6, STTR7, STTR9, and STTR10) had been previously described (Lindstedt et al., 2003; Lindstedt et al., 2004); five of these bear a high copy number of repeats in the five genomes (Table 2). STTR3 is a composite locus with 27-bp and 33-bp repeats, and STTR7 contains imperfect repeats; alleles of STTR3 and STTR7 are more appropriately represented by the repeat array sizes rather than the numbers of repeat units. Sixteen of the 39 loci displayed polymorphism in a panel of 40 diverse isolates tested and were further evaluated with panels of isolates with various genetic diversity.

3.2. Evaluation of 16 VNTRs

The 16 VNTRs that displayed polymorphism in a panel of 40 diverse isolates in an earlier test were analyzed on a total of 386 isolates. These VNTRs had between 2 and 22 alleles (Table 3). Six VNTRs (STTR3, STTR5, STTR6, STTR7, STTR9, and STTR10) displayed five or more alleles. The 386 isolates were divided into a P183 panel that consisted of 183 diverse isolates and a P203 panel that consisted of 203 closely related isolates. The allelic diversity values for P183 ranged from 0.011 to 0.922 (Table 3). STTR5, STTR10, STTR6, and STTR3 were the four most diverse loci, and ST23, ST20, ST28, and ST17 ranked as the fifth to the eighth diverse loci. STTR9 ranked as the tenth. The diversity values the VNTRs in the P183 isolates were correlated to the numbers of alleles (R^2 = 0.7724). Ten of the 16 loci displayed

variations in the P203 isolates. The diversity values for the VNTRs in the P203 isolates ranged from 0.009 to 0.721 and were correlated to the number of alleles in the 386 isolates (R^2 = 0.8957). The diversity values for the VNTRs in both panels were correlated with each other (R^2 = 0.8698). STTR6, STTR10, STTR5, and ST17 were the four most variable loci; STTR9, ST23, ST20, and STTR3 ranked as the fifth to the eighth in the P203 isolates.

3.3. Comparison of discriminatory power for PFGE and MLVAs

The levels of discriminatory power for PFGE and MLVA assays based on various combinations of VNTRs were assessed with the P183 panel of diverse isolates and the P203 panel of closely related isolates. PFGE discriminated the P183 isolates into 147 types, with a high level of discriminatory power (DI = 0.9956) (Table 4). MLVA4, an assay based on the four most diverse loci (STTR3, STTR5, STTR6 and STTR10), obtained 157 types, 10 more than PFGE. MLVA5, an assay based on STTR3, STTR5, STTR6, STTR9 and STTR10, obtained 159 types. MLVA8 and MLVA16 assays displayed a similar level of discriminatory power to MLVA4 and MLVA5. Thus, PFGE and various MLVA assays exhibited a high level of discriminatory power for the P183 panel of diverse isolates, although the MLVA assays obtained more genotypes than PFGE.

MLVA4 on the P203 isolates, based on the four most diverse (variable) loci (ST17, STTR5, STTR6 and STTR10), displayed remarkable resolving power. The MLVA4 assay obtained 108 types for the isolates (Table 4). The MLVA5 obtained the same number of types as the MLVA4, while MLVA8 obtained nine more types than the MLVA4. The remaining eight low-variability loci made little contribution to the discrimination of the closely related isolates.

3.4. Evaluation of MLVA on isolates from outbreaks

The 16 VNTR loci were analyzed in 54 *S.* Typhimurium isolates recovered from seven foodborne disease outbreaks that occurred from 1998 to 2003 (Table 5). The PFGE results revealed that isolates from an outbreak shared one or two PFGE types; isolates for outbreaks 1 and 2 were detected with two PFGE types. The predominant JPX.0001 type for outbreaks 1 and 2 was also the genotype for outbreaks 3 and 4. Only a single MLVA type was detected in isolates for each of the outbreaks (Table 5). Outbreaks 1, 2, 3 and 4, which occurred in a 2.5-year period, shared a common MLVA type, SM16.130. Only one PFGE type or one MLVA type was detected in isolates for each of the outbreaks 5, 6 and 7.

3.5. Phylogenetic analysis

The phylogenetic tree established using the MLVA16 data for the P203 isolates presented distinct clusters (Figure 1A). The genetic relationships among the isolates were in agreement with established with PFGE patterns (Figure 1B). Cluster A consisted of 94 MLVA16 types for 139 isolates, which fell into PFGE types, JPX.0001 (53 isolates), JPX.0010 (15), JPX.0013 (21), JPX.0077 (11), and JPX.0084 (39). The isolates for each of the PFGE types in cluster A were resolved, respectively, into 40, 14, 14, 10, and 31 MLVA16 types. The MLVA16 types for isolates with each of the PFGE types in cluster A were closely related and mixed mutually. A few isolates with different PFGE patterns shared common MLVA16 types (Figure 1A). Cluster B contained MLVA16 types, with a predominant type, for the 25 JPX.0143 isolates, while cluster C consisted of 17 types for the 19 JPX.0049 isolates and cluster D included two types, with a predominant type, for the 20 JPX.0559 isolates. PFGE patterns for each of the clusters were compared with those in the Danish database.

The PFGE patterns for the isolates in cluster A matched the patterns for strains with phage type DT104 in the Danish database, while the PFGE patterns for the isolates in clusters B, C and D matched the patterns for strains with phage types DT193, DT8, and DT120, respectively (Figure 1A).

4. Discussion

It is necessary to explore as many VNTRs as possible from the genomic sequence(s) of an organism in development of an MLVA method. Five *S.* Typhimurium genomic sequences were available at the time, and we used the VNTRDB computer program (Chang et al., 2007) to search tandem repeat (TR) loci from each sequence. Each of the five genomic sequences served, in turn, as the subject for the exploration of the TRs. In this study, VNTRDB ran for seven days to search thousands of TRs, located and counted the number of repeats for the TRs among the five genomic sequences; 39 loci were identified that displayed differences in the numbers of repeats among the five genomes (Table 2). Highly variable VNTRs are useful markers for discriminating closely related isolates, whereas loci having various degrees of variability are applicable to assess genetic relatedness among isolates with different evolutionary distances (Chiou et al., 2009; Keim et al., 2004). Theoretically, a larger set of VNTRs would be more favorable to obtain a clearer separation of distinct phylogenetic groups, so it is better to discover all of the possible VNTRs in developing an MLVA method. Because the five *S.* Typhimurium genomic sequence strains are genetically distant, many low-variability VNTRs can be discovered. Most of the 39 VNTRs had a low level of variability, as shown by the results from a preliminary evaluation with a panel of 40 diverse isolates. Only 16 of the 39 VNTRs were polymorphic in the 40 diverse isolates.

The 16 VNTRs were evaluated with a panel of 183 diverse isolates (P183) and a panel of 203 closely-related isolates (P203) to assess the level of diversity and the variability (or mutability) of the loci. The allelic diversity values for VNTRs are determined by the number and distribution frequency of alleles across a population (Keim et al., 2004). Consistently, STTR3, STTR5, STTR6, and STTR10, which have a

high number of alleles, displayed high diversity values across the P183 population (Table 3). In contrast, ST28 had only two alleles, but its diversity value was close to 0.5, greater than STTR7, which had five alleles across the population. The discrepancy between ST28 and STTR7 was due to an even distribution of the two ST28 alleles but an uneven distribution of the five STTR7 alleles in the population.

The diversity values obtained from the P203 closely related isolates represent the variability of the loci. Therefore, the results indicate that STTR5, STTR6, and STTR10 are hypervariable. Five VNTRs with diversity values between 0.028 and 0.096 are considered to be relatively variable (Table 3). An MLVA assay based on these highly variable VNTRs exhibits high resolving power for closely related isolates. This evaluation on the P203 isolates also indicates that STTR7 is not a highly variable locus, even though it exhibits a high copy number of repeats in the five sequenced *S.* Typhimurium strains (Table 2). STTR7 has a long repeat (39 bp), and some of its alleles contain imperfect repeat that resulted from an insertion or deletion of a few base pairs rather than by an intact repeat change within the repeat array region.

The results in Table 4 indicate that PFGE and various MLVAs, which were based on various combinations of VNTRs, exhibited high discriminatory power for the diverse P183 isolates. However, MLVA4, which was based on the four most highly variable VNTRs, obtained 10 more types than PFGE (Table 4). MLVA5 displayed a similar resolving power to the MLVA4 for the P183 diverse isolates. An MLVA4 assay also displayed a superior discrimination in the P203 closely related isolates; it obtained 108 types for the P203 isolates, which were randomly selected from isolates with eight prevalent PFGE types. With four additional loci compared to MLVA4, MLVA8 obtained nine more types than MLVA4. Therefore, the MLVA4 possesses

extreme resolving power for the PFGE-indistinguishable isolates, and the MLVA8 contributes considerable additional discriminatory power for the closely related isolates. Since four and eight VNTRs can be analyzed in one and two multiplex PCR reactions, respectively, MLVA4 and MLVA8 can be a high-throughput and cost-effective subtyping tool for routine analysis of *S.* Typhimurium isolates for epidemiological investigation and disease surveillance.

Among the 16 VNTRs, an MLVA5 assay based on STTR3, STTR5, STTR6, STTR9 and STTR10 has been adopted in many European laboratories for routine analysis of *S.* Typhimurium isolates. This study shows that the MLVA5 assay exhibited a level of resolution close to that of the MLVA4 assay in both panels of isolates with different diversity levels (Table 4). The MLVA5 protocol has been demonstrated to be superior to PFGE for surveillance and outbreak investigations of *S.* Typhimurium infection (Torpdahl et al., 2007). Since the MLVA5 protocol has been optimized in a multiplex PCR reaction and has been widely used in many laboratories for years, this protocol can be recommended to supplement PFGE in routine subtyping of isolates for surveillance of *S.* Typhimurium infection.

VNTRs may evolve rapidly (Noller et al., 2006; Vogler et al., 2006; Vogler et al., 2007); therefore, the stability of loci is a major concern in the use of MLVA. The stability of the five VNTRs STTR3, STTR5, STTR6, and STTR10 has been evaluated with 190 isolates from eight *S.* Typhimurium outbreaks and 15 isolates from seven patients (Hopkins et al., 2007); small changes were observed in the loci, but the VNTR profiles were stable during the course of the outbreaks. In this study, we analyzed 54 isolates recovered from seven outbreaks; isolates from each of the outbreaks were detected with a single MLVA type (Table 5). The isolates from the first four outbreaks,

occurring in a 2.5-year period, shared a common MLVA type, suggesting that the 16 VNTRs, including the three hypervariable loci, are relatively stable for the use of outbreak investigation.

Alleles at each locus are generally designated by the number of repeat units; this nomenclature is independent of the equipment used for fragment analysis and of the primers used. Among the VNTRs, STTR3 is a composite locus with 27- and 33-bp repeats. A three-digit nomenclature has been proposed for this locus; the first digit is for the number of 27-bp repeats, and the second and third digits are for the number of 33-bp repeats (Larsson et al., 2009). However, this nomenclature is ineffective when alleles of STTR3 bear imperfect repeats. In this study, we found 14 isolates out of the 440 total isolates tested that contained an allele with two 27-bp and seven 33-bp repeats but that had a deletion of 9 bp within the repeat array region. At a locus with a long repeat array, such as STTR3 or STTR7, indels should be common. We therefore recommend using the sizes of the repeat arrays to designate STTR3 and STTR7 alleles; this nomenclature is also independent of the method used for fragment analysis.

MLVA based on a small set of highly variable VNTRs, e.g., four VNTRs, can possess extreme resolving power for isolates, in particular closely-related isolates. MLVA is therefore a useful molecular tool for fine typing of isolates for outbreak investigation and disease surveillance. In addition, MLVA based on a larger set of VNTRs can also be a useful tool to establish phylogenetic relationships among isolates. The phylogenetic tree established using the MLVA16 data for the P203 isolates presented four distinct clusters. PFGE patterns for each of the four clusters matched strains with a unique phage type in the Danish database (Figure 1A). Thus,

each of the four MLVA clusters could represent a clone of a phage type. According to this study, DT104 clone is the most prevalent and may account for half of the *S.* Typhimurium isolates recovered in 2004–2008 in Taiwan.

S. Typhimurium is the second-most common *Salmonella* serovar in Taiwan, accounting for 21.7% of the 13,063 *Salmonella* isolates collected in 2004–2008, a proportion similar to the most prevalent serovar, *S.* Enteritidis (25.8%). Although more than 460 PFGE types were found in the 2,840 *S.* Typhimurium isolates, the genotypes were distributed unevenly. For example, the most prevalent type (JPX.0001) accounted for 33% of the *S.* Typhimurium collection, and the five most prevalent types made up 51% of the collection. Each of the five most prevalent PFGE types was detected almost every month during 2004–2008, so it is unlikely that they had a common source. In contrast, PFGE types JPX.0143 and JPX.0559 emerged more recently; they probably originated from a common source. In this study, isolates of each of the prevalent PFGE types were discriminated into many fine MLVA types (Figure 1A). The results indicate that PFGE is not discriminatory enough for *S.* Typhimurium isolates recovered in an endemic area of *S.* Typhimurium infection, e.g., Taiwan. In contrast, isolates with JPX.0559 and JPX.0143 had a predominant MLVA16 type; they could be associated with ongoing outbreaks during that period of time. Type JPX.0559 emerged in 2008 and has become a predominant type in 2009 in eastern Taiwan. JPX.0143 emerged in 2006 and predominated in 2007. In an endemic area of *S.* Typhimurium infection, MLVA must be implemented for the purposes of outbreak investigation and disease surveillance.

In conclusion, in silico exploration of five sequenced *S.* Typhimurium genomes identified 39 VNTRs. Most of the loci displayed low diversity; only 16 VNTRs

displayed polymorphism in a panel of 40 diverse isolates tested. Evaluations of the 16 VNTRs with panels of isolates of different genetic diversity levels revealed the degrees of allelic diversity of the VNTRs and indicated three hypervariable VNTRs, STTR5, STTR6 and STT10. MLVA assays based on a small set of loci, for example MLVA4 and MLVA5, can exhibit high resolving power for discriminating among isolates, in particular closely related isolates. A phylogenetic tree established using the MLVA16 profiles for the panel of closely related isolates presented four distinct clusters, which could be inferred to four different phage types. Therefore, MLVA based on a small set of highly variable VNTRs can exhibit a higher resolving power than PFGE and can be recommended to supplement PFGE in routine subtyping of isolates for outbreak investigation and disease surveillance. In addition, MLVA based on a larger set of loci is also a useful phylogenetic tool for *S.* Typhimurium.

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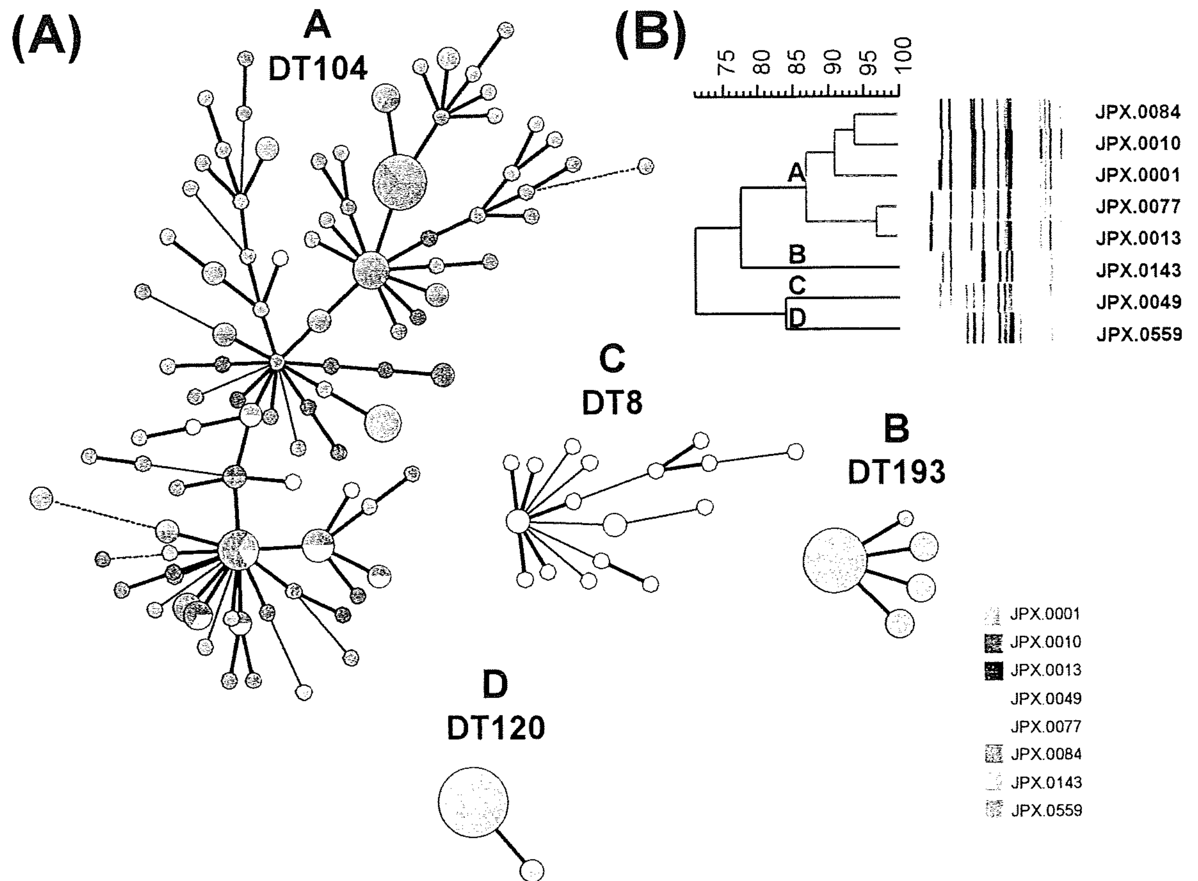

Figure 1. Phylogenetic tree established using MLVA16 profiles (A) and dendrogram established using PFGE patterns (B) for the P203 isolates. MLVA types for isolates with a common PFGE pattern are marked in the same color. Circle size is proportional to the number of isolates belonging to an MLVA type. Two or more MLVA types differing at three or fewer loci are regarded as a group and are marked in gray shadow. There are four MLVA clusters, A, B, C and D; each of the clusters consists of isolates with PFGE pattern(s) matching Danish strains belonging to a particular phage type, as indicated. Five PFGE patterns for isolates within cluster A share greater pattern similarity than clusters B, C and D.