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Genetic Diversity of *Actinobacillus pleuropneumoniae* Assessed by Amplified Fragment Length Polymorphism Analysis[▽]

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Amplified fragment length polymorphism (AFLP) was evaluated as a method for genotypic characterization and subtyping within the bacterial species *Actinobacillus pleuropneumoniae*. A total of 155 isolates of *A. pleuropneumoniae*, representing the serotypic variation described to occur within this species, were analyzed. In order to elucidate the species boundaries, six strains of the phylogenetically closely related species *Actinobacillus lignieresii* were also included. Furthermore, the ability of AFLP to subtype was studied using 42 isolates of serovar 2 and the performance compared to that obtained by pulsed-field gel electrophoresis (PFGE). AFLP analysis provided a clear separation of *A. lignieresii* and *A. pleuropneumoniae* and divided the isolates of *A. pleuropneumoniae* into 20 clusters. Most of the serovars of *A. pleuropneumoniae* were represented by single and quite homogeneous clusters. The exceptions were serovars 10, K2:O7, and K1:O7, which were represented by two clusters each. In the cases where the serovars were represented by more than one cluster, the existence of these clusters was supported by additional phenotypic or genotypic properties. Furthermore, AFLP typing was able to allocate serologically nontypeable isolates to appropriate genetic groups within the species. Further investigations are needed to determine whether some of the clusters revealed through AFLP analysis represent additional serovars. When evaluated as a method for subtyping within serovar 2 of *A. pleuropneumoniae*, AFLP was found to achieve a degree of separation among isolates superior to that obtained by PFGE. However, a higher degree of separation between serovar 2 isolates was obtained by a combination of the two methods.

Actinobacillus pleuropneumoniae is a gram-negative, encapsulated respiratory pathogen of swine and the causative agent of porcine pleuropneumonia (14). The disease occurs worldwide and has resulted in large economic losses to the swine industry. At present, 15 different serovars and two biotypes have been described (4). Serovar specificity is predominately due to structural differences in the capsular polysaccharides and for most serovars also differences in lipopolysaccharides (37). In some strains, an unusual pattern of surface antigens can occur, e.g., in strains having a capsular polysaccharide similar to that of serovar 2 and a lipopolysaccharide structure similar to that of serovar 7, which are designated K2:O7 (34). Virulence studies indicate considerable differences in virulence between serovars (7, 20, 40).

Serotyping has been the most commonly used technique for epidemiological monitoring of the disease in swine herds and for subsequent decisions on herd health status, prevention, therapy, and eradication. Many countries have long-term programs for targeting specific serovars of *A. pleuropneumoniae* (19). In Denmark, serological monitoring is a central part of the health classification in the specific-pathogen-free (SPF) system (26). In a geographically restricted area, usually a few serovars dominate. In North America, serovars 1, 5, and 7 are the most commonly isolated, whereas in Germany, serovars 2, 7, and 9 are the most prevalent (14). In Korea, serovars 2, 5, and 6 predominate (30), whereas in the United Kingdom, serovars 2, 3, and 8 are the most common (29). In Denmark, serovars 2, 5, and 6 account for approximately 94% of the strains isolated from swine with clinical diseases (22). However, due to the restricted numbers of serovars that tend to dominate in different regions, serotyping often fails to provide substantial epidemiological information.

Previous studies have shown that strains within a given serovar of *A. pleuropneumoniae* are genetically very similar, underlining that *A. pleuropneumoniae* seems to have a clonal population structure (32). Limited discrimination within a serovar was obtained using multilocus enzyme electrophoresis (MLEE) (31) and ribotyping (11). A higher level of discrimination within a serovar was obtained by pulsed-field gel electrophoresis (PFGE), which could subdivide 12 strains belonging to the same ribotype into seven distinct types (9).

A. pleuropneumoniae is genetically closely affiliated with *Actinobacillus lignieresii* (6, 25), a bacterium commonly found in the mucosal membranes of cattle and the cause of chronic infections in the tongue and jaw (tree tongue). These species can phenotypically be separated only on account of *A. pleuropneumoniae* being NAD dependent and hemolytic on blood agar (25). However, some strains of *A. pleuropneumoniae* are NAD independent (biovar 2) (39), and some strains of *A. pleuropneumoniae* are only weakly hemolytic. The animal species of isolation can be used as a supportive criterion for species identification; however, there exist a few reports on the isolation of *A. lignieresii* from swine as well (14). The 16S rRNA gene sequences of these two species differ by only 2 nucleotides (10), so their genotypic separation has been based on other genes, such as, for instance, *omlA* and *apxIV* (15, 41). So far, the genotypic relationship between these two species has not been thoroughly investigated.

Amplified fragment length polymorphism (AFLP) (46) is a

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highly versatile DNA fingerprinting method that is based on selective amplification of restriction fragments. AFLP has been widely used for identification of molecular markers in both eucaryotes and procaryotes for the purpose of genetic mapping (3, 28), differentiation of closely related organisms at the species and strain levels (27), detection of DNA polymorphisms in genome evolution studies (23), bacterial taxonomy (21), outbreak investigations (24, 44), and genome-wide expression analysis (2, 43). Until now, AFLP has not been evaluated for any species belonging to the genus *Actinobacillus*. In the present investigation, the method was used for analysis of *A. pleuropneumoniae* and *A. lignieresii* strains in order to elucidate the genotypic relationship between these two species as well as to investigate the intraspecies diversity of *A. pleuropneumoniae*. Furthermore, the utility of AFLP and PFGE for typing of *A. pleuropneumoniae* isolates belonging to serovar 2 was also evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Actinobacillus* strains used for this study are listed in Table 1. In addition to 16 reference strains representing serovars 1 to 15 (including serovars 5A and 5B), 93 Danish field isolates of *A. pleuropneumoniae*, all originating from different herds, were included in the study. These isolates represent serovars 1 ($n = 6$), 2 ($n = 33$), 5 ($n = 10$), 6 ($n = 9$), 7 ($n = 7$), 8 ($n = 3$), 10 ($n = 4$), 12 ($n = 8$), K1:O7 ($n = 6$), and K2:O7 ($n = 5$) and serologically nontypeable isolates ($n = 2$). In addition, one isolate of serovar 8 from the United Kingdom and two Canadian isolates of serovar K1:O7 were included. Out of the 33 isolates representing serovar 2, 20 isolates originated from nine cases of putative transmission of disease between Danish SPF herds. Six of these cases of transmission have earlier been investigated by ribotyping (11). In addition, eight serovar 2 strains from Canada, Hungary, Norway, Switzerland, and The Netherlands, which were earlier investigated by PFGE (9), were also included. To investigate the stability of the AFLP fingerprints, 26 additional isolates of serovar 10 and 9 serologically nontypeable isolates, all collected during a 3-month period from the same herd (45), were included. All together, a total of 155 isolates of *A. pleuropneumoniae* were included in the study. In order to evaluate the species boundaries, six strains of the phylogenetically closely related species *A. lignieresii* were analyzed. These included the reference strain ATCC 49236^T as well as five field isolates from Belgium, Denmark, Scotland, the United States, and Zimbabwe (Table 1).

All isolates of *A. pleuropneumoniae* were grown on PPLO agar (Difco), whereas all isolates of *A. lignieresii* were grown on Columbia agar supplemented with 5% bovine blood. All strains were incubated at 37°C overnight in atmospheric air.

Serotyping and PCR typing of *A. pleuropneumoniae*. All the Danish field isolates of *A. pleuropneumoniae* were serotyped by a latex agglutination test (12). Each isolate was tested with latex particles coated with polyclonal antibodies produced against whole cells of reference strains of *A. pleuropneumoniae* representing serovars 1 through 15. When the latex agglutination test showed a cross-reaction between different serovars, the isolates were also tested by immunodiffusion or indirect hemagglutination to determine the serovar (33).

Characterization of strains by serovar-specific PCR tests and by PCR typing based on the *apx* and *omlA* genes was performed, following methods published earlier (16, 22, 42). Typing of strains belonging to serovars 1 and 7 was performed using a *cps*-based PCR test developed by Angen et al. (1) (the primer sequences and PCR procedure are available on request).

AFLP analysis. Bacterial genomic DNA was extracted by using an EasyDNA kit (Invitrogen) according to the manufacturer's instructions. Five microliters of DNA samples, containing approximately 300 to 500 ng of genomic DNA, was digested with 10 U each of the EcoRI and BspDI restriction enzymes (New England Biolabs) in a restriction buffer containing 10 mM Tris-acetate (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate, 5 mM dithiothreitol, and 50 ng of bovine serum albumin per ml (46). The total reaction volume was 20 μl. Digestion was carried out in a water bath at 37°C for 2 h. Subsequently, 5 μl of digested genomic DNA was mixed with 15 μl of a ligation mixture containing 2 pmol of a double-stranded EcoRI adapter consisting of oligonucleotide sequences 5′-A ATTGCAAGAGCTCTCCAGTAC-3′ and 5′-TAGTACTGGAGAGCTCTTG C-3′, 20 pmol of a double-stranded BspDI adapter consisting of oligonucleotide sequences 5′-CGGACTAGAGTACACTGTC-3′ and 5′-GATCGACAGTGTA CTCTAGTC-3′, 1 U of T4 DNA ligase (Amersham), 2 μl of 10× ligase buffer (supplied with the enzyme), and 8 μl of the restriction buffer. Ligation was carried out overnight at room temperature.

Modified genomic restriction fragments were amplified by using nonselective EcoRI (5′-GGAGAGCTCTTGCAATTG-3′) and BspDI (5′-GTGTACTCTAG TCCGAT-3′) primers. The EcoRI primer was labeled at the 5′ end with 6-carboxyfluorescein dye. The amplification was performed with a final volume of 50 μl. The reaction mixture contained 2 μl of a 10-fold-diluted ligation product, 200 μM of each of the four deoxyribonucleoside triphosphates, 2.5 mM $MgCl_2$, 20 pmol EcoRI primer, 20 pmol BspDI primer, 1.5 U of AmpliTaq DNA polymerase (Applied Biosystems), 500 mM KCl, and 100 mM Tris-HCl (pH 8.3). The amplification was performed with a programmable thermal cycler by using an initial denaturation step at 94°C for 3 min followed by 30 cycles consisting of denaturation at 94°C for 60 s, annealing at 54°C for 60 s, and extension at 72°C for 90 s. The final cycle included a 10-min extension step at 72°C.

Amplification products were detected with an ABI 377 automated sequencer (Perkin Elmer) according to the manufacturer's instructions. Data collection, preprocessing, fragment sizing, and pattern analysis were done by using GeneScan 3.1 software (Applied Biosystems). Numerical analysis was performed using BioNumerics 4.1 software (Applied Maths). Levels of similarity between fingerprints were calculated by using the Dice similarity coefficient (S_D). Clustering of fingerprints was performed with the unweighted-pair group method using average linkages (UPGMA).

PFGE. The preparation of bacterial DNA for PFGE was performed as described by Olsen et al. (35). DNA embedded in agarose plugs was digested with the restriction enzyme SpeI (New England Biolabs) for 4 h according to the manufacturer's instructions. Electrophoresis was performed for 24 h at 14°C using the CHEF Bio-Rad DRIII electrophoresis system at 12 V cm^{-1}. The program used a pulse time starting with 1 s and increasing to 10 s after 24 h. Gels were stained in ethidium bromide solution and photographed under UV light. Gel photographs were subsequently scanned and analyzed with BioNumerics 4.1 (Applied Maths). The band patterns in the range of 20 to 200 kb were compared using S_D and UPGMA clustering.

The discriminatory powers of both AFLP and PFGE were calculated by using Simpson's index of diversity (18).

RESULTS

General features and reproducibility of the AFLP fingerprints. Amplification of EcoRI-BspDI DNA templates with AFLP primers yielded banding patterns consisting of approximately 90 fragments in the size range of 50 to 500 bp. In order to assess the reproducibility of the obtained profiles, 10 randomly selected samples were analyzed in duplicates. The repeated analysis was performed with fresh subcultures of the selected strains and included all steps of the AFLP assay. For all strains tested, identical banding patterns were obtained in the two runs, and only slight variations in band intensities were observed.

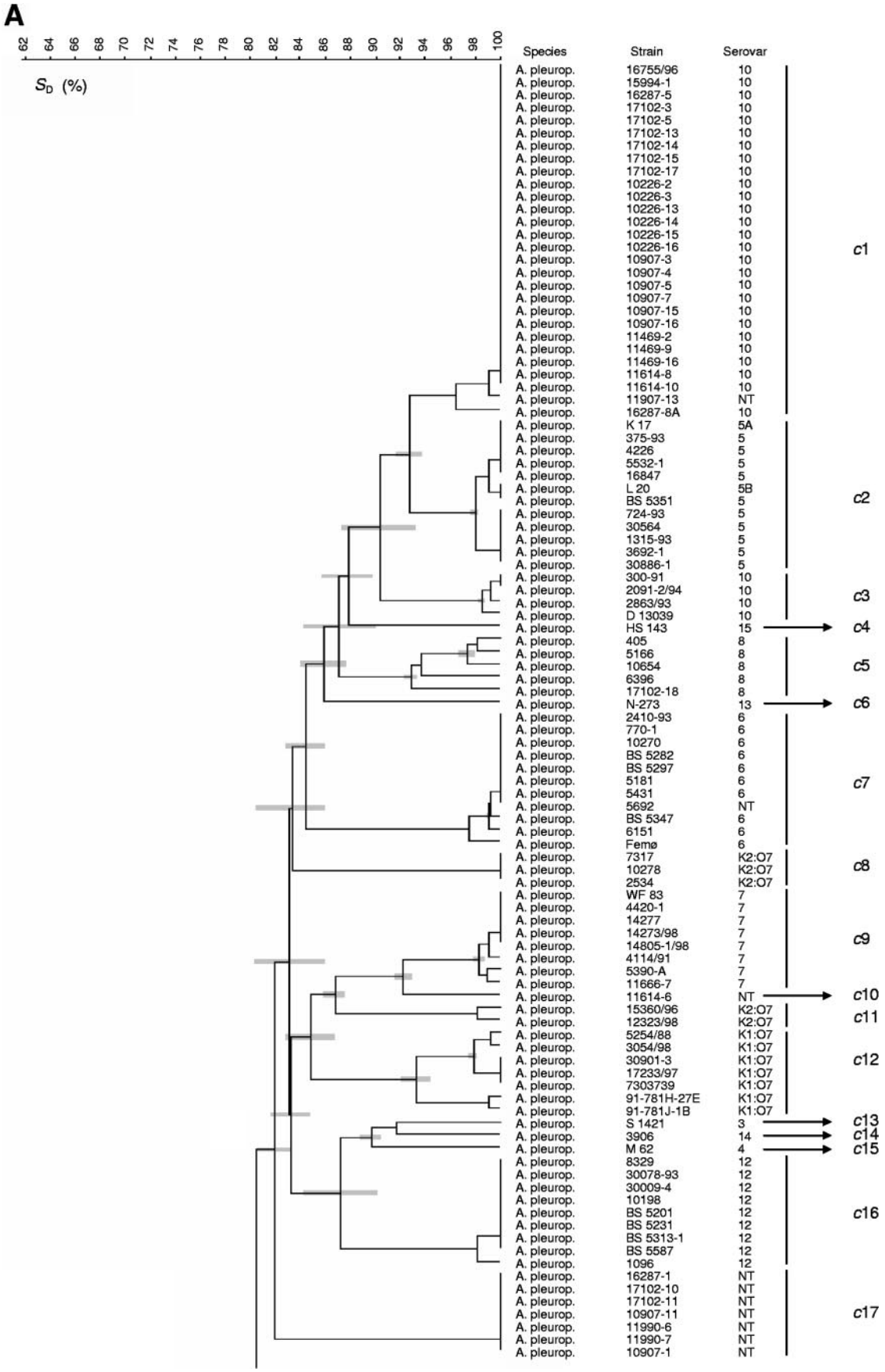
Relationship between *A. lignieresii* and *A. pleuropneumoniae* strains. In this study, AFLP was used for characterization of 161 *A. lignieresii* and *A. pleuropneumoniae* strains. Numerical analysis of AFLP data revealed two clearly distinguishable, species-specific clusters, with an overall similarity of 61.8% (Fig. 1B). The six analyzed *A. lignieresii* strains showed six different AFLP patterns, forming a cluster at a linkage level of 80.3%. The 155 analyzed *A. pleuropneumoniae* strains revealed 71 different AFLP profiles, with an overall similarity of 80.6%. The discriminatory index for *A. pleuropneumoniae* was 0.96.

The *A. pleuropneumoniae* cluster was divided into two subgroups, A and B, which were formed at linkage levels of 82% and 86%, respectively (Fig. 1A and B). Strains of *A. pleuropneumoniae* were further divided into 20 clusters or branches (*c*1 to *c*20 in Fig. 1A and B). With a threshold of 93%, each cluster essentially corresponded to the serovar of the isolates.

TABLE 1. *Actinobacillus pleuropneumoniae* and *Actinobacillus lignieresii* strains analyzed in this study

Strain designation(s)[a]	Serovar[b]	Origin	Note
A. pleuropneumoniae			
Shope 4074^T	1	Argentina	Reference strain
1536	2	Switzerland	Reference strain
S 1421	3	Switzerland	Reference strain
M 62	4	United States	Reference strain
K 17	5A	United States	Reference strain
L 20	5B	United States	Reference strain
Femø	6	Denmark	Reference strain
WF 83	7	Canada	Reference strain
405	8	Irland	Reference strain
13261	9	The Netherlands	Reference strain
D 13039	10	Denmark	Reference strain
56153	11	The Netherlands	Reference strain
8329	12	Denmark	Reference strain
N-273	13	Hungary	Reference strain
3906	14	Denmark	Reference strain
HS 143	15	Australia	Reference strain
4475; 5689; 10356/94; 3162/93; 608/94; 4987/93	1	Denmark	Field isolates
4226; 1198/93; 671-6; 427; BS5125; BS5199; 11302; 15332; 12798-1; 14476; 15427; 16257; 7503826	2	Denmark	Field isolates
30011-2; 30009-1	2	Denmark	Field isolates[c]
1010-20; 830-9	2	Denmark	Field isolates[c]
KB 47; GSP 27	2	Denmark	Field isolates[c]
752-1; 751-22	2	Denmark	Field isolates[c]
31661; 31738	2	Denmark	Field isolates[c]
30680; 21802-1; 21822-1	2	Denmark	Field isolates[c]
12783/98; 13843/98; 15609/98	2	Denmark	Field isolates[c]
7403410; 7503209	2	Denmark	Field isolates[c]
7504039; 7504439	2	Denmark	Field isolates[c]
1141-2; 2993-2	2	Canada	Fussing (9)
Hg84	2	Norway	Fussing (9)
4257	2	Switzerland	Fussing (9)
1604; 3619	2	The Netherlands	Fussing (9)
U21; U35	2	Hungary	Fussing (9)
375-93; 724-93; 30564; 1315-93; BS5351; 4226; 5532-1; 16847; 3692-1; 30886-1	5	Denmark	Field isolates
2410-93; 770-1; 10270; BS5282; BS5297; BS5347; 6151; 5181; 5431	6	Denmark	Field isolates
14277; 5390-A; 4114/91; 14273/98; 4420-1; 11666-7; 14805-1/98	7	Denmark	Field isolates
17102-18; 10654; 5166	8	Denmark	Field isolates
6396	8	United Kingdom	Field isolate
300-91; 2863/93; 2091-2/94; 16755/96	10	Denmark	Field isolates
15994-1; 16287-5; 17102-3; 17102-5; 17102-13; 17102-14; 17102-15; 17102-17; 10226-2; 10226-3; 10226-13; 10226-14; 10226-15; 10226-16; 10907-3; 10907-4; 10907-5; 10907-7; 10907-15; 10907-16; 11469-2; 11469-9; 11469-13; 11614-8; 11614-10; 16827-8A	10	Denmark	Field isolates[d]
1096; 30078-93; 30009-4; 10198; BS5201; BS5231; BS5313-1; BS5587	12	Denmark	Field isolates
7303739; 30901-3; 17233/97; 5254/88; 3054/98; 15053-6	K1:O7	Denmark	Field isolates
91-781H-27E; 91-781J-1B	K1:O7	Canada	Field isolates
7317; 10278; 2534; 15360/96; 12323/98	K2:O7	Denmark	Field isolates
17102-10; 17102-11; 10907-1; 10907-11; 10907-13; 11990-6; 11990-7; 11614-6; 16287-1	NT	Denmark	Field isolates[d]
5779/98; 5692	NT	Denmark	Field isolates
A. lignieresii			
ATCC 49236^T		United States	Bovine isolate
P 671		Denmark	Human isolate
Ac 3		Zimbabwe	Bovine isolate
B 96/11		Belgium	Bovine isolate
NCTC 4191		United States	Bovine isolate
CCUG 22227		Scotland	Ovine isolate

[a] All but underlined strains of serovar 2 were analyzed by PFGE.
[b] NT, serologically nontypeable isolates.
[c] Serovar 2 isolates from SPF herds (bold type) and neighboring conventional herds from nine cases of putative transmission of the disease.
[d] Isolates obtained from a single herd during a 3-month period (45).


A
62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100
S_D (%)
Species Strain Serovar
A. pleurop. 16755/96 10
A. pleurop. 15994-1 10
A. pleurop. 16287-5 10
A. pleurop. 17102-3 10
A. pleurop. 17102-5 10
A. pleurop. 17102-13 10
A. pleurop. 17102-14 10
A. pleurop. 17102-15 10
A. pleurop. 17102-17 10
A. pleurop. 10226-2 10
A. pleurop. 10226-3 10
A. pleurop. 10226-13 10
A. pleurop. 10226-14 10
A. pleurop. 10226-15 10
A. pleurop. 10226-16 10
A. pleurop. 10907-3 10
A. pleurop. 10907-4 10
A. pleurop. 10907-5 10
A. pleurop. 10907-7 10
A. pleurop. 10907-15 10
A. pleurop. 10907-16 10
A. pleurop. 11469-2 10
A. pleurop. 11469-9 10
A. pleurop. 11469-16 10
A. pleurop. 11614-8 10
A. pleurop. 11614-10 10
A. pleurop. 11907-13 NT
A. pleurop. 16287-8A 10
A. pleurop. K 17 5A
A. pleurop. 375-93 5
A. pleurop. 4226 5
A. pleurop. 5532-1 5
A. pleurop. 16847 5
A. pleurop. L 20 5B
A. pleurop. BS 5351 5
A. pleurop. 724-93 5
A. pleurop. 30564 5
A. pleurop. 1315-93 5
A. pleurop. 3692-1 5
A. pleurop. 30886-1 5
A. pleurop. 300-91 10
A. pleurop. 2091-2/94 10
A. pleurop. 2863/93 10
A. pleurop. D 13039 10
A. pleurop. HS 143 15
A. pleurop. 405 8
A. pleurop. 5166 8
A. pleurop. 10654 8
A. pleurop. 6396 8
A. pleurop. 17102-18 8
A. pleurop. N-273 13
A. pleurop. 2410-93 6
A. pleurop. 770-1 6
A. pleurop. 10270 6
A. pleurop. BS 5282 6
A. pleurop. BS 5297 6
A. pleurop. 5181 6
A. pleurop. 5431 6
A. pleurop. 5692 NT
A. pleurop. BS 5347 6
A. pleurop. 6151 6
A. pleurop. Femø 6
A. pleurop. 7317 K2:O7
A. pleurop. 10278 K2:O7
A. pleurop. 2534 K2:O7
A. pleurop. WF 83 7
A. pleurop. 4420-1 7
A. pleurop. 14277 7
A. pleurop. 14273/98 7
A. pleurop. 14805-1/98 7
A. pleurop. 4114/91 7
A. pleurop. 5390-A 7
A. pleurop. 11666-7 7
A. pleurop. 11614-6 NT
A. pleurop. 15360/96 K2:O7
A. pleurop. 12323/98 K2:O7
A. pleurop. 5254/88 K1:O7
A. pleurop. 3054/98 K1:O7
A. pleurop. 30901-3 K1:O7
A. pleurop. 17233/97 K1:O7
A. pleurop. 7303739 K1:O7
A. pleurop. 91-781H-27E K1:O7
A. pleurop. 91-781J-1B K1:O7
A. pleurop. S 1421 3
A. pleurop. 3906 14
A. pleurop. M 62 4
A. pleurop. 8329 12
A. pleurop. 30078-93 12
A. pleurop. 30009-4 12
A. pleurop. 10198 12
A. pleurop. BS 5201 12
A. pleurop. BS 5231 12
A. pleurop. BS 5313-1 12
A. pleurop. BS 5587 12
A. pleurop. 1096 12
A. pleurop. 16287-1 NT
A. pleurop. 17102-10 NT
A. pleurop. 17102-11 NT
A. pleurop. 10907-11 NT
A. pleurop. 11990-6 NT
A. pleurop. 11990-7 NT
A. pleurop. 10907-1 NT
c1
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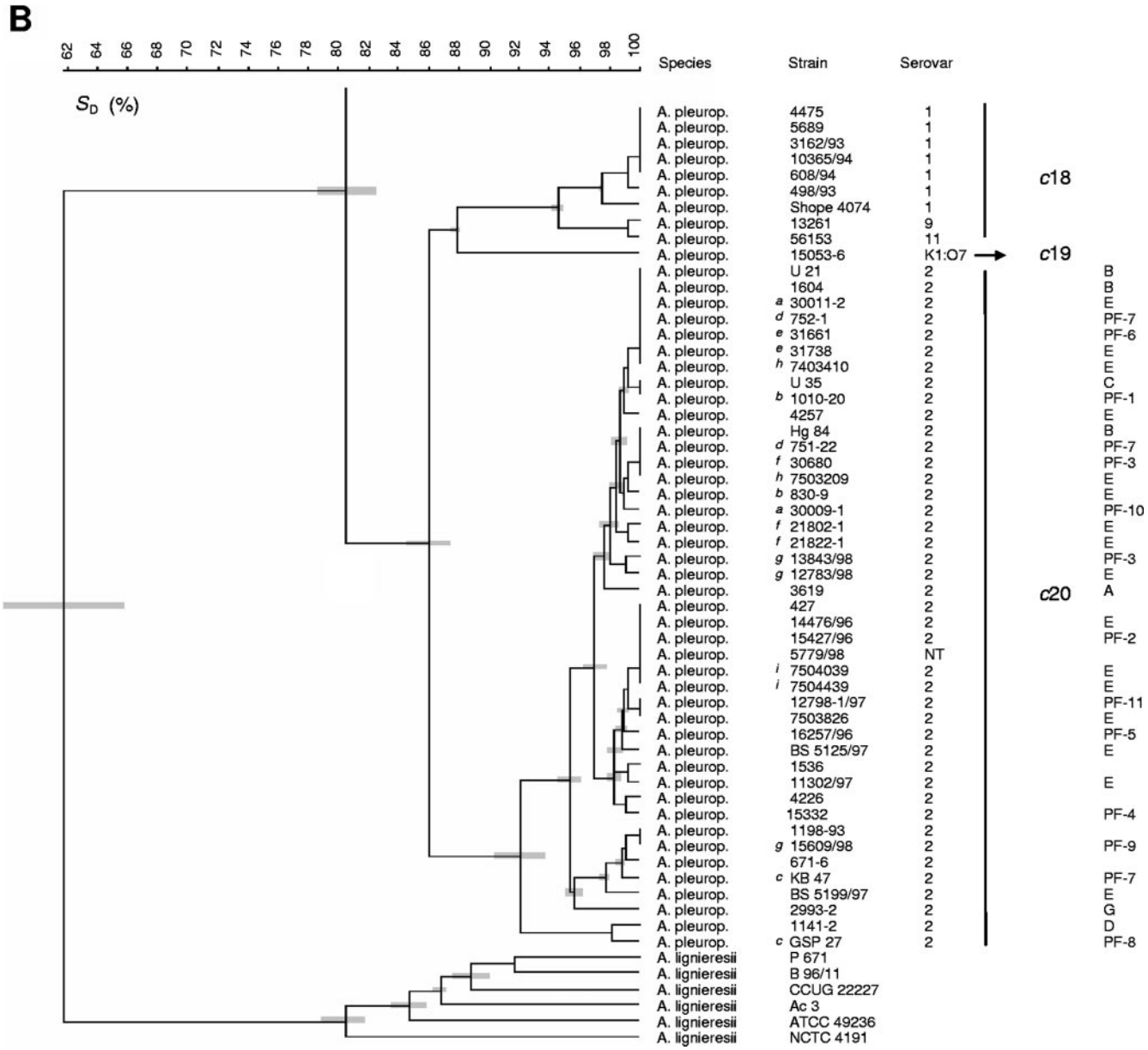

FIG. 1. Relatedness between *A. pleuropneumoniae* and *A. lignieresii* isolates as inferred from their AFLP profiles. Similarity was calculated by using S_D, and the dendrogram was produced by using UPGMA. *c*1 to *c*20, distinct clusters and branches obtained by comparison of AFLP data; A to E, PFGE types detected earlier and defined by Fussing (9); PF-1 to PF-11, PFGE types detected in this study; *a* to *i*, serovar 2 isolates from SPF herds and neighboring conventional herds from nine cases of putative transmission of the disease.

The only exception was cluster *c*18, which contained strains of serovar 1 as well as the reference strains of serovars 9 and 11. Subgroup A (Fig. 1A) included strains belonging to serovars 5, 6, 7, 8, 10, 12, K1:O7, K2:O7, and 10 of the serologically nontypeable isolates. This subgroup also included serovars 3, 4, 13, 14, and 15, which were represented by a single reference strain. Subgroup B (Fig. 1B) included strains belonging to serovars 1 and 2 as well as two single representatives of serovars 9 and 11. This subgroup included also a single strain having serovar K1:O7 (branch *c*19) and a single strain that was serologically untypeable (cluster *c*20).

Each of the reference strains of serovars 1, 2, 3, 4, 6, 8, 9, 10, 11, 13, 14, and 15 showed a unique AFLP fingerprint, while the reference strains for serovars 5A, 5B, 7, and 12 shared common AFLP profiles, with four, one, four, and seven field isolates, respectively. Different degrees of genomic diversity were detected within different *A. pleuropneumoniae* serovars. The lowest levels of genomic diversity were found among strains belonging to serovars 1, 5, 6, 7, and 12, whose overall AFLP profile similarities were 97.5%, 98%, 97.5%, 98.3%, and 98.1%, respectively. Moderately higher levels of diversity were detected among the strains belonging to serovars 2, 8, and 10, which showed overall AFLP profile similarities of 92.1%, 93%, and 92.3%, respectively. The highest levels of genomic diver-

sity were observed within serovars K2:O7 and K1:O7, which had overall AFLP profile similarities of 83.6% and 86%, respectively. All strains of a given serovar were found in the same cluster, except for serotypes 10, K2:O7, and K1:O7, which were represented by two clusters each.

Comparison of the AFLP profiles of 11 serologically nontypeable isolates revealed their affiliations to different serovars. Two of these isolates, 5779/98 and 5692, showed AFLP profiles that were identical to the profiles of five serovar 2 and seven serovar 6 isolates, respectively. The allocations of these two strains to their respective serovars were confirmed using a serovar-specific PCR test (22). The nine remaining serologically nontypeable isolates were isolated from the same herd during a 3-month period. Seven of these isolates showed indistinguishable AFLP profiles (cluster *c*17) (Fig. 1A). One serologically untypeable isolate was closely related (99% AFLP profile similarity) to a group of 26 AFLP-indistinguishable serovar 10 strains, which were also isolated from the same herd during a 3-month period (cluster *c*1) (Fig. 1A), while 1 isolate showed an AFLP profile that was most similar (92.2% similarity) to serovar 7 strains (branch *c*10) (Fig. 1A).

Discriminatory power of AFLP within *A. pleuropneumoniae* serovar 2. Among the 43 analyzed serovar 2 strains, including the nontypeable serovar-2-like isolate 5779/98, 26 unique AFLP profiles were detected. The discriminatory index for serovar 2 was 0.95. Numerical analysis of AFLP data showed four discrete groups, at linkage levels of 97.5%, 98.3%, 95.7%, and 92.1% (cluster *c*20) (Fig. 1B). The grouping did not correlate with the spatial or temporal origins of the strains, i.e., strains isolated from neighboring herds over a short period of time showed different AFLP profiles, while strains isolated several years apart at distant geographical localities showed identical AFLP fingerprints. Strains U35 (Hungary), 1141-2 (Canada), 4257 (Switzerland), and 2993-2 (Canada), having PFGE types C, D, E, and G, respectively (9), showed different AFLP profiles. Strains U21 (Hungary) and 1604 (The Netherlands), having PFGE type B, showed indistinguishable AFLP profiles, which were, however, different from the AFLP profile of strain Hg84 (Norway), which had PFGE type B as well (9). Distinct AFLP profiles were also obtained from reference strain 1536 (Switzerland) and strain 3619 (The Netherlands), both having PFGE type A.

Twenty serovar 2 isolates originated from nine cases of putative transmission of disease between Danish SPF herds and neighboring conventional herds (Table 1). Thirteen of these isolates were earlier analyzed by ribotyping (11). In only two cases did an isolate obtained from an infected SPF herd show an AFLP profile identical to the profile of an isolate obtained from a corresponding neighboring herd. In a single case, the isolates obtained from an SPF herd and a corresponding neighboring herd had highly similar (99% similarity) AFLP profiles (cluster *c*20) (Fig. 1B). In six cases, the AFLP profile of an isolate derived from an SPF herd was different (92.3 to 97.3% similarity) from the profile obtained from an isolate obtained from a neighboring herd. However, the analysis also showed that three epidemiologically unrelated isolates (30011-2, 752-1, and 31661) obtained from different SPF herds had identical AFLP profiles. Two other isolates (1010-20 and 30680), which also originated from different SPF herds, had AFLP profiles identical to those of the epidemiologically unrelated strains U35 (from Hungary) and Hg84 (from Norway), respectively.

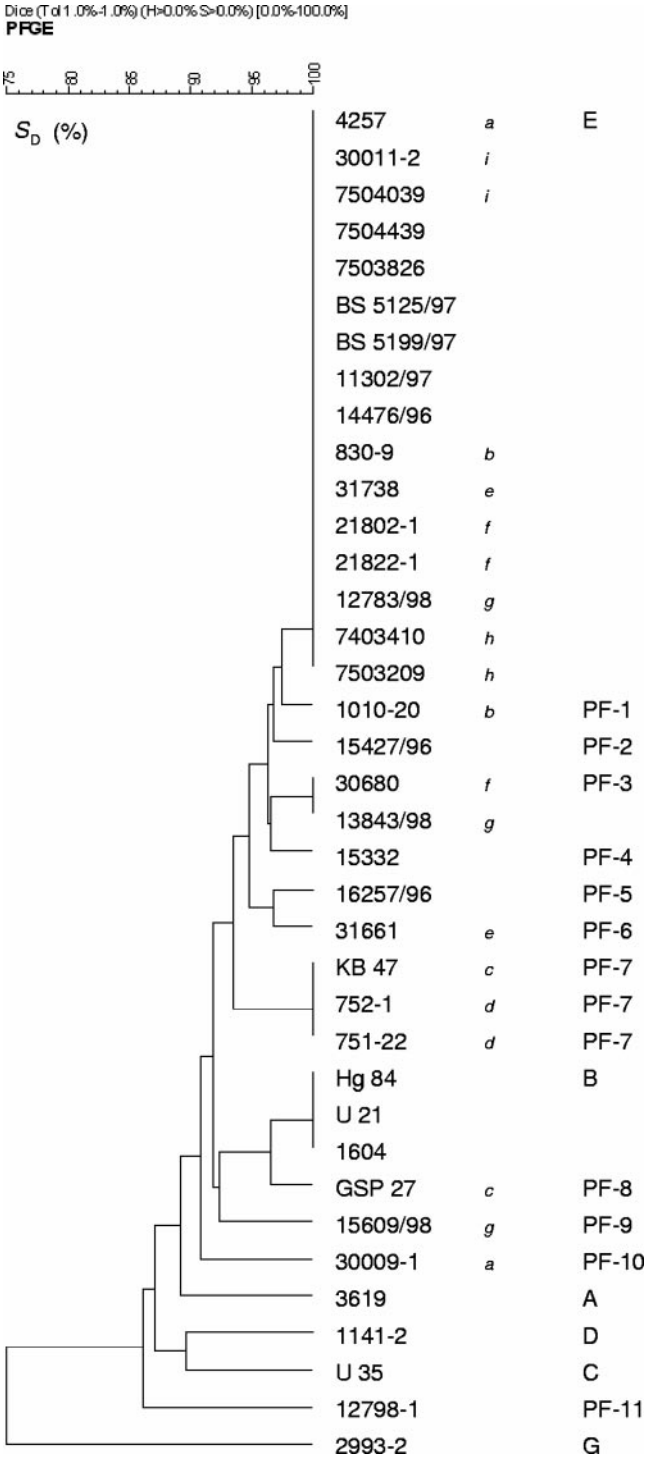

FIG. 2. Relatedness among serovar 2 isolates as inferred from PFGE profiles produced after digestion with the SpeI restriction enzyme. A to E, PFGE types detected earlier and defined by Fussing (9); PF-1 to PF-11, PFGE types detected in this study; *a* to *i*, serovar 2 isolates from SPF herds and neighboring conventional herds from nine cases of putative transmission of the disease.

PFGE separation within *A. pleuropneumoniae* serovar 2. Among 37 serovar 2 isolates that were analyzed by PFGE in this

study (Table 1), 17 different pulsed-field (PF) types, having an overall similarity of 75%, were detected (Fig. 2). The discriminatory index of PFGE was 0.81. Eight isolates earlier investigated by PFGE using the same restriction enzyme were allocated to PF types as reported by Fussing (9). Fifteen Danish isolates and strain 4257 from Switzerland shared a common PF type that was earlier designated type E (9). Danish field isolates 30680 and 13843/98 also shared a common PF type (PF-3), as did isolates KB47, 752-1, and 751-22 (PF-7) (Fig. 2). Strains U21 (Hungary) and 1604 (The Netherlands) and five Danish field isolates, which all shared a common AFLP profile, showed four distinct PF types (B, E, PF-6, and PF-7) (Fig. 1B and 2). Isolates Hg84 (Norway), 7503209, 30680, and 751-22 (Denmark), having identical AFLP profiles, showed PF types B, E, PF-3, and PF-7, respectively. Isolates 14476/96 (PF type E) and 15427/96 (PF-2) also shared a common AFLP profile, as did isolates 12789-1/97 and 7503826, having PF-11 and PF type E, respectively.

DISCUSSION

The AFLP method used in this study possesses several properties that are desirable for an efficient typing method, i.e., universal typeability, high reproducibility, and high discriminatory power. All strains investigated were found to be typeable, and a sufficient number of bands were obtained to give a high discriminatory index. The reproducibility of the method was also found to be high despite the minor variation observed upon analysis of identical samples. These variations consisted exclusively of differences in band intensities (i.e., the heights of the peaks), which presumably occurred due to slight variations in amplification efficiency and/or variances in loading of samples in the detection step, but they were insufficient to alter the conclusion made regarding the interstrain relationships.

The stability of the typing method was assessed using two groups of isolates (clusters *c*1 and *c*17) (Fig. 1A) collected from the same herd, from six sows and their piglets, during a 3-month period (45). Cluster *c*17 comprised seven serologically nontypeable strains, all showing indistinguishable AFLP profiles, while cluster *c*1 comprised 27 isolates from this herd, of which all but 1 (11907-13) were found to be typeable as serovar 10. Among these 27 isolates, all but 2 (11907-13 and 16287-8A) had identical AFLP patterns. The AFLP profiles of isolates 11907-13 and 16287-8A differed from the AFLP profile that was common for the remaining isolates of the clusters at 1 and 10 positions, respectively. The rather close genetic relatedness of isolate 11907-13 and the other serovar 10 strains in cluster *c*1 indicates that they likely present the same clonal line in spite of small differences in their AFLP profiles. This seems not to be the case with isolate 16287-8A, whose more pronounced AFLP polymorphism could not likely be explained by alteration in genetic composition in a short time frame. The two different clones of serovar 10 were probably introduced to this herd by the sows. Interestingly, cluster *c*1 comprised also isolate 16755/96, which was obtained from a different herd several months earlier than the remaining strains of the cluster. This indicates that the AFLP pattern remains stable in a clonal line of *A. pleuropneumoniae* replicating in a host animal.

A clear separation between the strains of *A. pleuropneumoniae* and *A. lignieresii* was found. This provides additional evidence that these two species represent distinct genetic entities, in spite of their high phenotypic similarity and close phylogenetic relationship as evaluated by 16S rRNA gene sequencing (6, 10), and is in concordance with previous observations concerning the ability of AFLP to distinguish isolates within a given genus to the level of a species (27, 36).

Earlier investigations of *A. pleuropneumoniae* by MLEE have shown that the species has a predominantly clonal population structure (31, 32). A similar conclusion could be deduced from the results of the present investigation, where most of the serovars were represented by single and relatively homogeneous clusters. In this study, genetic homogeneity was particularly high among isolates of serovars 1, 5, 6, 7, and 12. Chatellier et al. (5) have also reported substantial genetic homogeneity among Canadian isolates of serovar 5 collected over a 14-year period that could not be differentiated by means of random amplified polymorphic DNA analysis and showed only minor variations in their PFGE profiles, while genetic homogeneity among isolates of serovar 6 were demonstrated by Møller et al. (31). However, Chatellier et al. (5) found a more pronounced diversity within serovar 1, while in the study of Møller et al. (31), isolates of serovars 7 and 12 showed more genetic diversity than those of the remaining serovars. Hampson et al. (17) also found limited diversity among isolates of serovars 3 and 7 by using MLEE. The low levels of diversity within serovars 1, 7, and 12 found by AFLP analysis in this study are probably due to the limited number of isolates analyzed as well as the fact that the isolates were derived from a restricted geographic region. Analysis of isolates derived from distant geographical regions and obtained over larger time frames may reveal a better overview of genetic diversity within these serovars.

The AFLP analysis revealed a more pronounced genetic diversification within serovars 10, K2:O7, and K1:O7. Genetic diversity within serovar 10 has not been reported earlier. Strains of this serovar were found in two clusters: *c*1, representing isolates from two different Danish herds, and *c*3, including the serovar reference strains as well as three Danish field isolates from different herds. All isolates of both clusters were found to have identical *apx* and *omlA* profiles when investigated by the PCR typing method described by Gram et al. (16). However, most of the isolates of cluster *c*1 have earlier been reported to show serological cross-reactions to serovar 3 (45). Further investigations are needed in order to determine whether the isolates found in cluster *c*1 represent a new serological variety within *A. pleuropneumoniae*.

Strains representing serovar K2:O7 (34) were found in clusters *c*8 and *c*11 (Fig. 1A). The genetic heterogeneity among these strains was confirmed by testing these strains with PCR tests targeting the *cps* genes. The three strains of cluster *c*8 gave rise to amplicons similar in size to those from serovar 2 (22) and serovar 8 (42), whereas the two strains of cluster *c*11 gave rise to the species-specific *omlA* amplicon only (22).

Genetic heterogeneity was also found within serovar K1:O7 (13), represented by cluster *c*12 and branch *c*19. The seven strains of cluster *c*10 all produced amplicons of the same size as those from serovar 1 when the *cps*-based PCR test described by Angen et al. (1) was used, whereas the single strain on branch *c*19 produced an amplicon of the same size as that from serovar 7 in the same PCR assay. Furthermore, the *apx* genes of the two clusters also differed, i.e., the single strain on branch

*c*19 had an *apx* profile similar to that of serovar 10, whereas the strains of cluster *c*12 had *apx* profiles similar to those of serovars 7 and 12.

Eleven serologically nontypeable isolates were also included in the investigation. Seven of these isolates showing indistinguishable AFLP profiles (*c*17) (Fig. 1A) were isolated from the same herd (45) and likely represent a single clone. The genetic compositions of these isolates are apparently quite different from those of any other known serovar, which exemplifies that diversity within the species exceeds the genetic boundaries associated with the known serovar. One serologically nontypeable isolate (5779/98) was found in cluster *c*20, together with all isolates representing serovar 2, and another (5692) was found in cluster *c*7, representing serovar 6. Interestingly, both isolates were typeable as serovars 2 and 6, respectively, when the *cps*-based PCR test of Jessing et al. (22) was used, indicating that they represent capsuleless varieties of the organism. One nontypeable isolate (11907-13) was found in cluster *c*1, together with isolates of serovar 10. The nontypeable isolate originated from the same herd as 26 other isolates of this cluster. The last nontypeable isolate (11614-6) was located as a single strain on branch *c*10, most closely related to serovar 7 strains. This isolates differed, however, from the serovar 7 isolates of cluster *c*9 by not producing any serovar 7-specific amplicon in the *cps*-based PCR test of Angen et al. (1).

At the arbitrarily chosen level of 93% similarity, which was used as threshold for cluster definition in this study, only one cluster was heterogeneous regarding the serovars of the strains, i.e., cluster *c*18, containing strains of serovar 1 as well as the reference strains of serovars 9 and 11. This finding is in accordance with previous investigations where a close relationship with regard to *apx* toxins (8), *omlA* types (16), and surface antigens (37) has been shown between these three serovars.

In summary, we have found that all the clusters revealed through analysis of the AFLP patterns represent subpopulations within the species *A. pleuropneumoniae* corresponding generally to the serovars of the isolates. In the cases where the serovars are represented by more than one cluster, the existence of these clusters is supported by additional phenotypic or genotypic properties. Furthermore, AFLP typing was able to allocate serologically nontypeable isolates to appropriate genetic groups within the species. Further investigations are needed to determine whether some of the clusters revealed through the AFLP analysis represent additional serovars within the species.

In Denmark, serovar 2 accounts for approximately 60% of the strains isolated from swine with clinical disease. Consequently, a high degree of separation between isolates of this serovar is of great importance for epidemiological purposes. Møller et al. (31) analyzed 250 isolates, 212 of these originating from Denmark, by MLEE, dividing the isolates into 35 distinct electrophoretic types (ETs). The 110 isolates belonging to serovar 2 were divided into four different ETs, with one of the ETs representing 106 of the isolates, resulting in a low level of discrimination by MLEE within serovar 2. Fussing et al. (11) evaluated ribotyping in an epidemiological study of Danish isolates of *A. pleuropneumoniae*. The 220 isolates were divided into 26 distinct ribotypes. However, also here the discriminatory power within one serovar was rather low. Within serovar 2, there were found eight ribotypes among 105 isolates, with the two dominating ribotypes representing 91% of the isolates. A higher level of discrimination within a serovar was obtained by PFGE, which could subdivide 12 isolates belonging to the same ribotype into seven distinct PF types (9). Eight of these isolates, representing six of the PF types, were also available for this study. All of these isolates showed different AFLP profiles (Fig. 1B). Furthermore, AFLP provided additional separation among three isolates sharing PF type B and two isolates belonging to PF type A.

To further evaluate the performance of the two methods, we have comparatively analyzed 37 of the serovar 2 isolates. The discriminatory index for AFLP (0.95) was found to be higher than that for PFGE (0.83). However, on several occasions strains that could not be differentiated by AFLP showed slight differences in their PFGE profiles and vice versa. AFLP analysis of serovar 2 strains originating from cases of putative transmission of the disease between SPF and neighboring herds indicated possible transmission in three of nine cases. In one of these three cases, the corresponding strains also had identical PFGE profiles, while in two cases, the corresponding strains showed slight differences in their PFGE profiles. Furthermore, common AFLP profiles were also observed between isolates from Denmark, the United Kingdom, and Hungary. The existence of such widespread clones makes firm epidemiological conclusions difficult. This underlines the importance of a thorough investigation of the genetic variation within a bacterial population before conclusions about epidemiological relationships can be determined. These observations also indicate the high clonality within this serovar of *A. pleuropneumoniae*, which poses a great challenge for the development of an efficient subtyping system. Therefore, for the time, a combination of the two methods, supplemented with thorough epidemiological information, seems necessary for investigations of transmission of a disease caused by serovar 2.

In conclusion, the present investigation has shown that AFLP represents a valuable tool for typing of *A. pleuropneumoniae* isolates, regardless of the taxonomic level that it was applied to. A clear separation between the two affiliated species *A. lignieresii* and *A. pleuropneumoniae* was demonstrated. AFLP was found to separate the isolates into well-defined groups, generally corresponding to the serovars. AFLP could further be used for description of genotypic variation within some of the serovars and for successful classification of nontypeable isolates within the population of *A. pleuropneumoniae*.

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