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Genomic Relatedness of *Chlamydia* Isolates Determined by Amplified Fragment Length Polymorphism Analysis

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The genomic relatedness of 19 *Chlamydia pneumoniae* isolates (17 from respiratory origin and 2 from atherosclerotic origin), 21 *Chlamydia trachomatis* isolates (all serovars from the human biovar, an isolate from the mouse biovar, and a porcine isolate), 6 *Chlamydia psittaci* isolates (5 avian isolates and 1 feline isolate), and 1 *Chlamydia pecorum* isolate was studied by analyzing genomic amplified fragment length polymorphism (AFLP) fingerprints. The AFLP procedure was adapted from a previously developed method for characterization of clinical *C. trachomatis* isolates. The fingerprints of all *C. pneumoniae* isolates were nearly identical, clustering together at a Dice similarity of 92.6% (± 1.6% standard deviation). The fingerprints of the *C. trachomatis* isolates of human, mouse, and swine origin were clearly distinct from each other. The fingerprints of the isolates from the human biovar could be divided into at least 12 different types when the presence or absence of specific bands was taken into account. The *C. psittaci* fingerprints could be divided into a parakeet, a pigeon, and a feline type. The fingerprint of *C. pecorum* was clearly distinct from all others. Cluster analysis of selected isolates from all species revealed groups other than those based on sequence data from single genes (in particular, *omp1* and rRNA genes) but was in agreement with available DNA-DNA hybridization data. In conclusion, cluster analysis of AFLP fingerprints of representatives of all species provided suggestions for a grouping of chlamydiae based on the analysis of the whole genome. Furthermore, genomic AFLP analysis showed that the genome of *C. pneumoniae* is highly conserved and that no differences exist between isolates of respiratory and atherosclerotic origins.

Chlamydiae are obligate intracellularly growing bacteria. They are widespread throughout the world and infect both humans and animals. Currently, four species, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pecorum*, belonging to the genus *Chlamydia* of the family *Chlamydiaceae* within the order *Chlamydiales*, are recognized (10, 11, 35). *C. pneumoniae* and *C. trachomatis* are primarily human pathogens. *C. pneumoniae* has been recognized as a major cause of respiratory infections. In addition, *C. pneumoniae* infection has been associated with new-onset asthma, exacerbation of chronic asthma, atherosclerotic disease, and, recently, Alzheimer's dementia (2, 22). *C. trachomatis* is a major cause of sexually transmitted diseases and trachoma in humans (18). *C. psittaci* and *C. pecorum* are primarily animal pathogens, but *C. psittaci* may cause zoonotic infections (44).

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified genes has been used to characterize chlamydial isolates. Using PCR-RFLP analysis of different genes, *Chlamydia* could be differentiated at the species level, and *C. trachomatis* and *C. psittaci* could be differentiated at a strain level corresponding to serovars and types (4, 12–14, 24, 29, 31, 53). However, *C. pneumoniae* isolates originating from all over the world could not be differentiated by this technique (4, 13, 29). Furthermore, the available sequence data for *C. pneumoniae* shows complete or nearly complete conservation for *omp1*, *omp2*, 16S rRNA, domain I of the 23S rRNA, RNase P RNA, the genes for the 53-kDa protein and the 76-kDa protein, *dnaK*, and *waaA* (*kdtA*) and the 16S-23S ribosomal DNA intergenic spacer (7, 13, 15, 17, 21, 26, 30, 39, 40, 55).

By analysis of the whole genome using RFLP (1, 5, 9, 27, 28, 37, 38, 42), random amplification of polymorphic DNA (RAPD) (41, 45), or hybridization (5, 6, 9), the four species could be differentiated, and subgroups could be recognized within the *C. trachomatis* and *C. psittaci* species. These findings are in agreement with the power of discrimination of RFLP and RAPD at the species-to-strain level and of DNA-DNA hybridization at the genus-to-subspecies level (52). RFLP analysis of the genome of *C. pneumoniae* showed only two nearly identical patterns. One extra band was observed in two of eight *C. pneumoniae* isolates (5). However, DNA-DNA hybridization experiments showed 94 to 96% relatedness among *C. pneumoniae* isolates, suggesting at least some genomic variation (6).

Recently, a novel high-resolution technique has been introduced for whole-genome analysis: amplified fragment length polymorphism (AFLP) (54). This technique requires relatively low amounts of genomic DNA. The DNA is digested by a combination of a restriction enzyme that has a high number of restriction sites in DNA and a restriction enzyme that has an average number of restriction sites in DNA. Selected sets of restriction fragments are amplified and analyzed on gels. This technique has proven its usefulness as a tool in bacterial taxonomy and epidemiology (16, 20, 43) and has also been applied in *C. trachomatis* research (32).

Here, we report on the application of AFLP to analyze the differences among *Chlamydia* species and, within the species, among subgroups.

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TABLE 1. *Chlamydia* isolates used in the present study

Species	Isolate	Source[a]	Host	Clinical manifestation
C. pneumoniae	A-03	1	Human	Atheroma, coronary artery
	AR-39	2	Human	Respiratory tract infection
	AR-338	2	Human	Respiratory tract infection
	BAL-16	1	Human	Bronchoalveolar lavage, patient with AIDS
	CM-1	3	Human	Pneumonia
	CWL-011	3	Human	Pneumonia
	CWL-029	3	Human	Pneumonia
	CWL-050	3	Human	Pneumonia
	GRO-21	4	Human	Pneumonia
	H-12	5	Human	Respiratory tract infection
	IOL-207	6	Human	Conjunctivitis
	K-7	5	Human	Respiratory tract infection
	NWL-1	7	Human	Pneumonia
	P-1	5	Human	Respiratory tract infection
	PS-32	8	Human	Atheroma, carotid endarterectomy
	TW-183	2	Human	Conjunctivitis
	UZG-1	9	Human	Pneumonia
	2023	10	Human	Respiratory tract infection
	2043	10	Human	Pneumonia
C. trachomatis	A/Sa-1	11	Human	Trachoma
	B/TW-5	11	Human	Trachoma
	Ba/Apache-2	10	Human	Trachoma
	C/UW-1	11	Human	Trachoma
	D/IC-CAL-8	11	Human	Urogenital infection
	Da/MT-566	12	Human	Urogenital infection
	D-/NL-326	12	Human	Urogenital infection
	D′	13	Human	Urogenital infection
	E/DK-20	11	Human	Urogenital infection
	F/MRC-301	11	Human	Urogenital infection
	G/IOL-238	11	Human	Urogenital infection
	H/UW-4	11	Human	Urogenital infection
	I/UW-12	11	Human	Urogenital infection
	I′	13	Human	Urogenital infection
	J/UW-36	11	Human	Urogenital infection
	K/UW-31	11	Human	Urogenital infection
	L1/440-L	11	Human	Urogenital infection
	L2/434-B	11	Human	Urogenital infection
	L3/404-L	11	Human	Urogenital infection
	MoPn/Nigg II	10	Mouse	Pneumonitis
	R19	14	Swine	Pneumonia
C. psittaci	6BC	10	Parakeet	Psittacosis
	ORNI	15	Human	Psittacosis
	Cat12137	15	Cat	Conjunctivitis
	D661	15	Pigeon	Unknown cause of death
	P635	15	Parakeet	Healthy excretor
	P650	15	Parakeet	Healthy; owner had psittacosis
C. pecorum	E58 (McNutt)	10	Calf	Encephalitis

[a] The *Chlamydia* isolates were obtained from the following sources: 1, J. T. Summersgill, University of Louisville, Louisville, Ky.; 2, Washington Research Foundation, Seattle, Wash.; 3, C. M. Black, Centers for Disease Control and Prevention, Atlanta, Ga.; 4, S. Farholt, Statens Seruminstitut, Copenhagen, Denmark; 5, P. Saikku, University of Oulu, Oulu, Finland; 6, J. Treharne, University of London, London, United Kingdom; 7, M. W. Carter, PHLS Central Public Health Laboratory, London, United Kingdom; 8, C.-C. Kuo, University of Washington, Seattle; 9, M. van den Abeele, University Hospital, Ghent, Belgium; 10, American Type Culture Collection, Manassas, Va.; 11, S. Darouger, London, United Kingdom; 12, M. F. Lampe, University of Washington, Seattle; 13, J. H. T. Wagenvoort, Rotterdam, The Netherlands; 14, A. A. Andersen, National Animal Disease Center, Ames, Iowa; 15, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

MATERIALS AND METHODS

***Chlamydia* isolates.** The reference and laboratory isolates studied are summarized in Table 1. The species, serovar, and biovar information was obtained previously by established procedures (3, 10, 22).

Isolation and propagation of *Chlamydia* in cell culture. HeLa 229 (ATCC CCL 2.1) (for propagation of *C. psittaci* and *C. trachomatis*) and HEp2 (ATCC CCL 23) (for propagation of *C. pneumoniae* and *C. pecorum*) cell lines were maintained in Iscove's modified Dulbecco medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. Isolation of chlamydiae from clinical samples or mouse lung homogenates and propagation of *Chlamydia* isolates were carried out as described previously (29). All isolates were tested for the presence of *Mycoplasma* contamination by using a *Mycoplasma* group-specific PCR (34). When positive, the chlamydial isolates were decontaminated by Triton X-100 treatment as previously described (34) or by passage in mice via intranasal infection followed by reisolation from the lungs 3 days postinfection.

Preparation of genomic DNA. Elementary bodies (EBs) were harvested by sonicating cell monolayers from four shell vials with >75% infected cells in 1 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. Next, 114 μl of the EB preparation was digested with 120 μg of DNase I (Boehringer Mannheim, Mannheim, Germany) in 10 mM $MgCl_2$ for 5 h at 37°C. After inactivation of the DNase for 20 min at 70°C, RNA was digested with RNase A (Sigma, St. Louis, Mo.) at a concentration of 0.1 mg/ml. Chlamydial DNA was purified from the EBs with the High Pure PCR template preparation kit (Boehringer Mannheim) according to the instructions of the manufacturer. The DNA was eluted in 200 μl

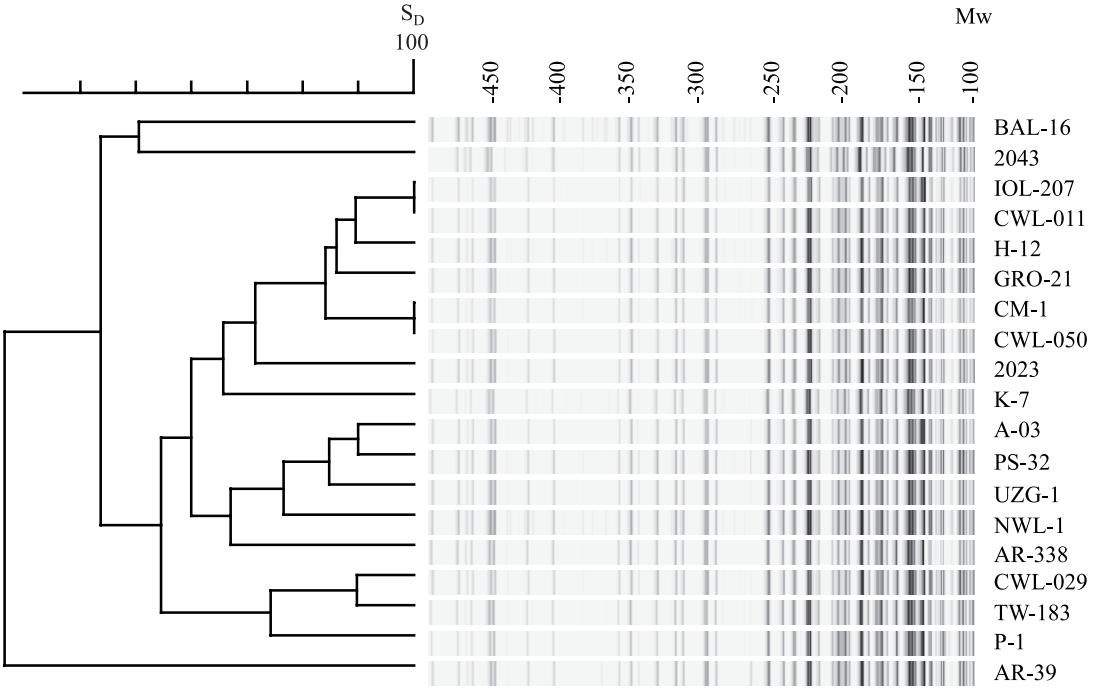

FIG. 1. Digitized AFLP fingerprints and phylogram of 19 *C. pneumoniae* isolates. The phylogram was inferred by the UPGMA method with a band-based Dice similarity coefficient (S_D) matrix. S_D values (in percentages) and molecular sizes (Mw) (in base pairs) are shown above the phylogram and fingerprints, respectively. The names of the isolates as presented in Table 1 are shown on the right.

of 10 mM Tris-HCl, pH 8.5, and tested for the absence of human DNA by a β-globin PCR assay as described previously (23).

AFLP analysis. Chlamydial DNA was digested with the restriction enzymes *Eco*RI (Pharmacia LKB Biotechnology, Uppsala, Sweden) and *Mse*I (New England Biolabs [NEB] Inc., Beverly, Mass.). Simultaneously, generally applicable double-stranded oligonucleotide adaptors, composed of a unique sequence and an overhang complementary to the restriction sites in the genomic digest as described previously (20, 32), were ligated to the restriction fragments for 3 h at 37°C. This mixture consisted of 4 μl of chlamydial DNA, 1 U of *Eco*RI, 1 U of *Mse*I, 2 pmol of *Eco*RI adaptor, 2 pmol of *Mse*I adaptor, 1 μl of ligase buffer (NEB) (50 mM Tris-HCl [pH 7.5] containing 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 25 μg of bovine serum albumin/ml), 0.5 M NaCl, 50 μg of bovine serum albumin (NEB)/ml, 0.6 U of T4 DNA-ligase (NEB), and H_2O up to 10 μl. After restriction and ligation, the DNA was diluted with H_2O to a final volume of 100 μl and stored at −20°C until it was further analyzed. The ligation products, now having unique sequences from the adaptors at both sites, were amplified by PCR with adaptor-specific primers. The PCRs were performed and optimized as described previously (20, 32). The primers had, in addition to the adaptor-specific sequence, no (Eco-0 and Mse-0) or one (Eco-A and Mse-C) additional selective nucleotide at the 3′-terminal end of the primer. This selective nucleotide allowed the amplification of only a subset of restriction fragments when the banding pattern, obtained after PCR with primers without a selective nucleotide, was too complex. The Eco-0 primer or the Eco-A primer was fluorescently labeled with Texas red (Isogen Bioscience BV, Maarssen, The Netherlands). The PCR products were separated in a denaturing polyacrylamide sequencing gel with a Vistra 725 automated sequencer (Amersham Life Science, Cleveland, Ohio). Fluoroimages of the banding pattern saved by the sequencer in a computer TIFF file were analyzed with GelCompar version 4.0 software (Applied Maths, Kortrijk, Belgium). The fluoroimages were normalized by alignment of the AFLP patterns by using molecular size markers included at regular intervals in each gel, and background fluorescence was subtracted with mathematical algorithms included in the GelCompar software. Fluorescent amplification fragments between 100 and 500 bp were included in the cluster analysis. Levels of correlation between fingerprints were calculated with the curve-based Pearson product moment correlation coefficient (46) and the band-based Dice similarity coefficient (S_D), which is equal to the ratio of twice the number of bands common for fingerprints A and B and the total number of bands in fingerprints A and B (46). To calculate S_D, bands were assigned to the fluoroimages automatically by the GelCompar software, using a minimal elevation of 5% of a band relative to the surrounding area and a minimal area of 0.5% of a band relative to the total area of the banding pattern, and to match bands in two compared fingerprints, a position tolerance of 0.8% relative to the total length of the pattern was allowed. Cluster analysis was carried out by the unweighted pair group method using arithmetic averages (UPGMA) algorithm (46) included in the GelCompar software.

DNA-DNA hybridization data. DNA-DNA hybridization data from previous studies (6, 9, 10) were used to infer a phylogram by the UPGMA method of the PHYLIP program package, version 3.5c (8). The final phylogram was visualized with the TreeView program, version 1.30 (36).

RESULTS

All chlamydial isolates were free of *Mycoplasma* contamination as determined by PCR, or, if positive, they were decontaminated by Triton X-100 treatment or mouse passage prior to AFLP analysis. All chlamydial DNA preparations were free of human DNA as assessed by the β-globin PCR assay. This ensures that the fingerprints obtained by AFLP analysis are specific for chlamydia.

Using a selection of *C. trachomatis* and *C. pneumoniae* isolates, the most discriminatory primer combination in the AFLP reaction was determined. The primer pair Eco-0–Mse-C was selected, since AFLP fingerprints of different *C. trachomatis* serovars showed different banding patterns (32). With a selection of *C. pneumoniae* isolates, no variation was observed in the AFLP fingerprints with either of the primer combinations. However, since the primer combination Eco-0–Mse-C showed a discrete banding pattern and a sufficient number of bands to be informative, and since different AFLP fingerprints were observed for closely related *C. trachomatis* isolates (32), this primer pair was used in all subsequent experiments.

The results of cluster analyses with matrices of Pearson product moment correlation coefficients and of Dice similarity coefficients were identical regarding the grouping of chlamydiae, as described below.

AFLP fingerprints of selected isolates with or without Triton X-100 treatment or mouse passage (*C. trachomatis* isolate R19 and *C. pneumoniae* isolates CWL-029, CWL-050, PS-32, and 2023) were identical regarding the number and location of

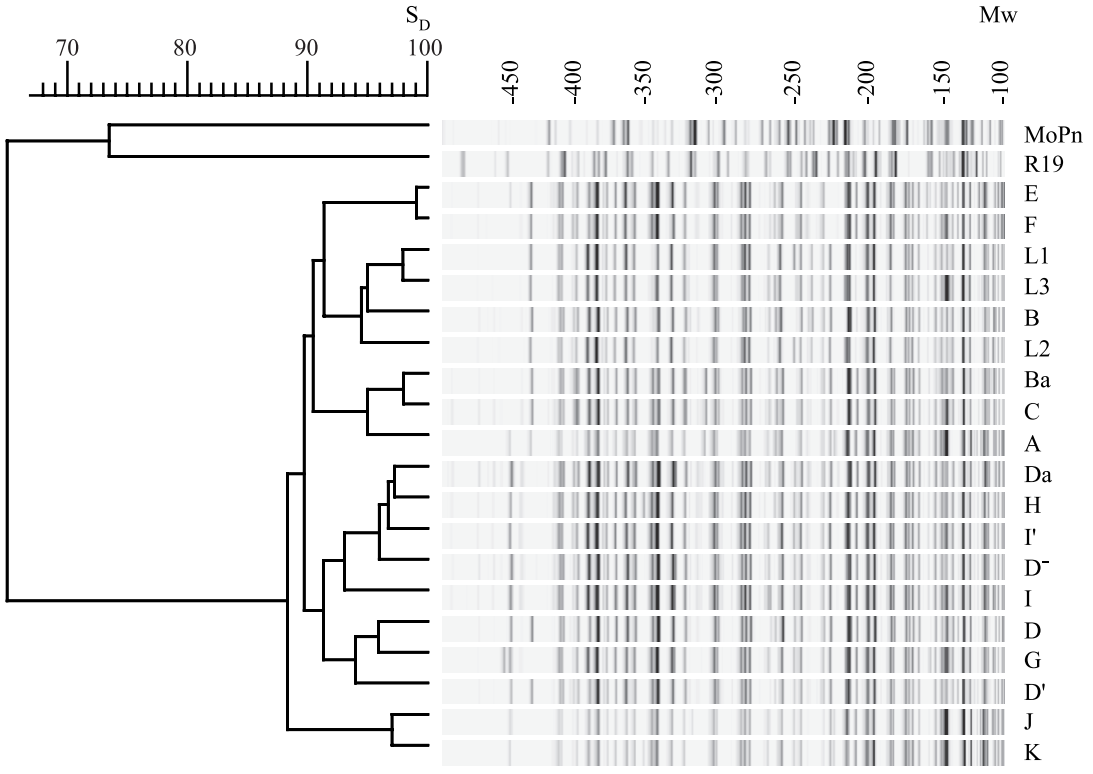

FIG. 2. Digitized AFLP fingerprints and phylogram of 21 *C. trachomatis* isolates. The phylogram was inferred by the UPGMA method with a band-based Dice similarity coefficient (S_D) matrix. S_D values (in percentages) and molecular sizes (Mw) (in base pairs) are shown above the phylogram and fingerprints, respectively. The names of the isolates as presented in Table 1 are shown on the right.

bands, clustering at an S_D of ≥96.8%. Only minor variation was observed in the intensities of some bands.

The AFLP fingerprints of all 19 *C. pneumoniae* isolates were nearly identical, clustering at an S_D of 92.6% (±1.6% standard deviation [SD]) (Fig. 1). Only minor variation in the intensities of some bands could be noted, while the number and location of all bands were identical.

The AFLP fingerprints of *C. trachomatis* showed considerable heterogeneity (Fig. 2). The mouse and swine isolates were different from the human isolates. The clusters separated from each other at an S_D of 64.9% (±2.4% SD) (Fig. 2). Also, the mouse and swine isolates were different from each other (S_D = 73.5%) (Fig. 2). The human isolates clustered at an S_D of 88.3% (±2.5% SD) (Fig. 2). Within the human cluster, at least 12 different fingerprint types could be observed, based on the number and locations of specific bands.

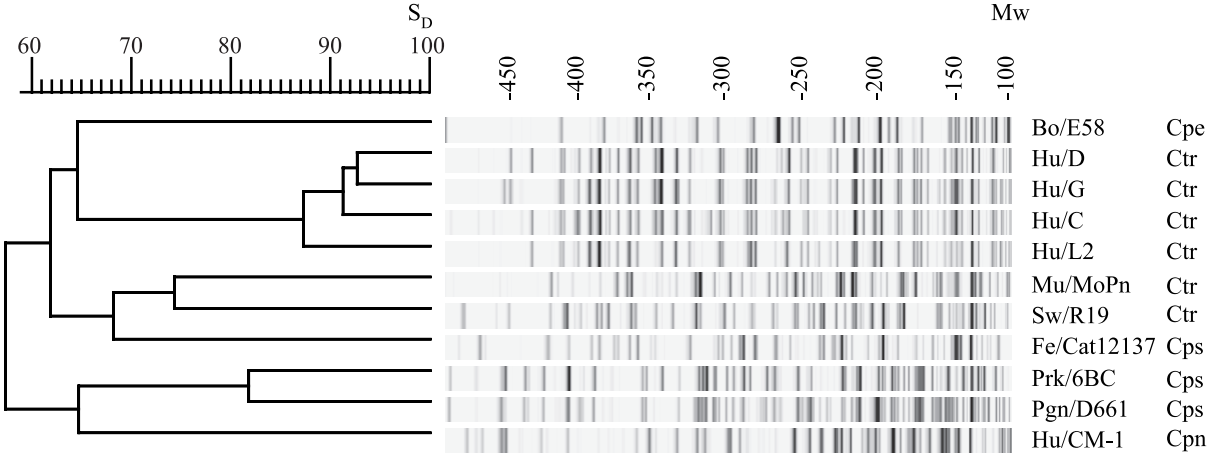

FIG. 3. Digitized AFLP fingerprints and phylogram of representative isolates of all four *Chlamydia* species. The phylogram was inferred by the UPGMA method with a band-based Dice similarity coefficient (S_D) matrix. S_D values (in percentages) and molecular sizes (Mw) (in base pairs) are shown above the phylogram and fingerprints, respectively. The names of the isolates as presented in Table 1 are shown on the right, with prefixes indicating the hosts (Bo, bovine; Fe, feline; Hu, human; Mu, murine; Pgn, pigeon; Prk, parakeet; Sw, swine) and species codes (Cpe, *C. pecorum*; Cpn, *C. pneumoniae*; Cps, *C. psittaci*; Ctr, *C. trachomatis*).

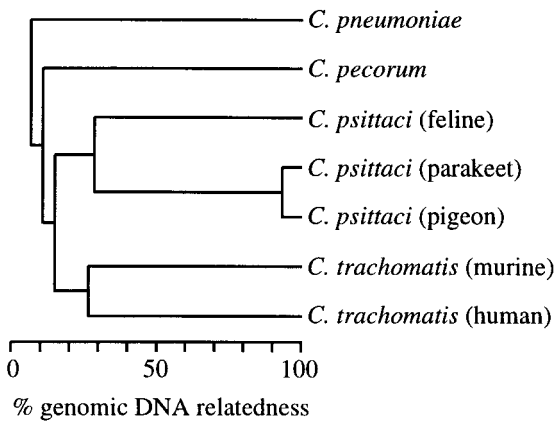

FIG. 4. Phylogram inferred by the UPGMA method with the DNA-DNA hybridization data matrix in Table 3. The relatedness between *C. pneumoniae* and *C. trachomatis* (murine) is missing and has been estimated as minimal (≤5%).

A cluster analysis with representatives of all species and some subgroups is shown in Fig. 3 for AFLP fingerprints and in Fig. 4 for DNA-DNA hybridization relatedness. The matrices of corresponding S_D values and previously published DNA-DNA hybridization relatedness percentages are summarized in Tables 2 and 3, respectively. Only one *C. psittaci* parakeet isolate was included in the cluster analysis of AFLP fingerprints, since *C. psittaci* isolates 6BC, ORNI, P635, and P650 were identical in the number, locations, and intensities of bands, clustering at an S_D of 95.4% (±1.2% SD) (data not shown). Within the species *C. psittaci*, three different AFLP fingerprints, a parakeet, a pigeon, and a feline type, could be observed, corresponding to the hosts from which the isolates originated. Regarding the human *C. trachomatis* isolates as one operational taxonomic unit (OTU), eight different OTUs could be recognized in the phylogram of AFLP fingerprint types (*C. pneumoniae*, human *C. trachomatis*, mouse *C. trachomatis*, swine *C. trachomatis*, feline *C. psittaci*, parakeet *C. psittaci*, pigeon *C. psittaci*, and *C. pecorum*) (Fig. 3). Using DNA-DNA hybridization data to infer a phylogram, six different OTUs could be recognized (*C. pneumoniae*, human *C. trachomatis*, mouse *C. trachomatis*, feline *C. psittaci*, parakeet and pigeon *C. psittaci*, and *C. pecorum*) (Fig. 4). These OTUs were identical to those of the corresponding AFLP fingerprint types, except for the *C. psittaci* isolates of parakeet and pigeon origin. These were regarded as one OTU based on their high relatedness (93.4%).

DISCUSSION

In this study, we demonstrated the use of AFLP for the analysis of chlamydial genomic DNA. The *C. pneumoniae* isolates, including respiratory and atherosclerotic isolates, showed identical AFLP fingerprints, while those of human *C. trachomatis* isolates could be divided into several groups. The AFLP fingerprints of *C. trachomatis* mouse and swine isolates were different from each other and from those of the human *C. trachomatis* isolates. AFLP fingerprints of *C. psittaci* isolates could be differentiated into three types, a parakeet, a pigeon, and a feline type. The AFLP fingerprint of the *C. pecorum* isolate was clearly distinct from those of the other *Chlamydia* species.

Some *Chlamydia* species or subspecies harbor plasmids of approximately 7.5 kbp that may interfere with the banding pattern of AFLP. Analysis of plasmid sequences derived from GenBank revealed that, theoretically, one or two bands in the fingerprint could originate from plasmids. Since plasmid sequences are highly conserved within human *C. trachomatis* and subgroups of *C. psittaci* (25, 49), the interference of plasmid-derived fragments in the cluster analysis was probably negligible.

The genome of *C. pneumoniae* is highly conserved, since the AFLP fingerprints of world-wide-derived isolates were almost identical, in agreement with previously reported genomic RFLP analysis results (5). However, considering the DNA-DNA hybridization relatedness of 94 to 96% among *C. pneumoniae* isolates (6), some differences might be expected. Other restriction enzyme combinations might improve the differentiation of *C. pneumoniae* isolates by AFLP analysis. Nevertheless, our results showed that isolates from atherosclerotic lesions are identical to those from the respiratory tract. These findings are in agreement with the reported sequences and Southern hybridization analysis data of these isolates (15, 30).

Three main AFLP fingerprint groups could be recognized within the species *C. trachomatis*, related to the host: human, mouse, and swine. This grouping is in agreement with data

TABLE 2. Relatedness of chlamydial genomic DNA as assessed by AFLP analysis

Isolate	Relatedness[a]									
	C. pneumoniae	*C. psittaci*			*C. trachomatis*					
							Human			
	Human CM-1	Parakeet 6BC	Pigeon D661	Feline Cat12137	Murine MoPn	Swine R19	C	D	G	L2
C. psittaci										
Parakeet/6BC	64.6									
Pigeon/D661	64.8	81.7								
Feline/Cat12137	57.8	58.0	56.9							
C. trachomatis										
Murine/MoPn	58.4	56.6	63.0	71.0						
Swine/R19	59.6	57.7	65.5	65.3	74.2					
Human/C	47.8	56.9	63.1	64.6	58.9	60.0				
Human/D	46.7	60.0	62.4	68.1	58.1	63.3	91.7			
Human/G	52.7	57.4	58.2	63.2	57.4	62.6	90.7	92.6		
Human/L2	53.9	54.5	53.7	60.2	60.9	61.9	88.4	86.0	87.2	
C. pecorum Bovine/E58	57.5	57.8	58.6	57.1	65.1	65.9	60.5	66.7	65.9	65.1

[a] Values represent percent similarity between AFLP fingerprints, calculated using the band-based Dice similarity coefficient.

TABLE 3. Relatedness of chlamydial genomic DNA as assessed by DNA-DNA hybridization analysis

Isolate	Relatedness[a]					
	C. pneumoniae[b]	*C. psittaci*			*C. trachomatis*	
		Parakeet 6BC	Pigeon[c]	Feline Pn-1	Murine MoPn	Human L2
C. psittaci						
Parakeet/6BC	≤6.0					
Pigeon[c]	≤5.5	93.4				
Feline/Pn-1	7.3	30.0	27.3			
C. trachomatis						
Murine/MoPn		4	11.5	5.5		
Human/L2	7	15	30.5	24	26.5	
C. pecorum Bovine/Maeda	10	10	10	10.5	10.5	21

[a] The values represent average percentages of DNA relatedness, based on data from references 6, 9, and 10.
[b] The data for *C. pneumoniae* were derived from isolates TW-183, AR-39, LR-65, and AR458 (6). The relatedness among *C. pneumoniae* isolates was ≥94% (6).
[c] The data for *C. psittaci* pigeon were derived from isolates Cal10/Mn and P1041, both of which had a pigeon restriction endonuclease fingerprint type (6, 9). The relatedness was ≥94% in this fingerprint type (9).

from other studies using different approaches (7, 9, 19, 40, 41). Among the human *C. trachomatis* isolates, at least 12 different AFLP fingerprint types could be recognized, despite a high relatedness in DNA-DNA hybridization of 92 to 100% (6, 9, 19). This observation is in agreement with results obtained with RAPD and RFLP analyses of genomic DNA (37, 38, 42, 45). However, subgroups of AFLP fingerprint types did not correlate with serogroups (33), biovar groups (lymphogranuloma venereum or trachoma), or *omp1* groups (47). It would be interesting to study correlations of AFLP fingerprint types with phenotypical or clinical features.

The *C. psittaci* isolates showed three AFLP fingerprints corresponding with their hosts, parakeet, pigeon, and cat, in agreement with previous reports based on other techniques (6, 9, 19, 40, 41, 48).

Our AFLP data support the species status of *C. pecorum* (10), in agreement with previous reports (7, 9, 19, 39, 40, 48).

No cutoff S_D value exists at which all isolates are clustered within one of the four currently recognized species. However, using the criterion of a cutoff S_D value of 80%, all isolates are clustered in seven groups, supporting the suggestion that at least seven groups within the genus *Chlamydia* should be recognized as species: *C. pneumoniae*, a human *C. trachomatis* group, a mouse *C. trachomatis* group, a swine *C. trachomatis* group, an avian *C. psittaci* group, a feline *C. psittaci* group, and *C. pecorum* (7, 9, 12, 40). Adding other isolates to the analysis, like *C. psittaci* abortion and guinea pig inclusion conjunctivitis isolates, might suggest even more groups. Using previously reported DNA-DNA hybridization data to infer a phylogram, the *C. psittaci* and *C. trachomatis* isolates clustered in two distinct clusters, in agreement with sequence data (7, 19, 39, 40, 48). However, when using the criterion of 50% or greater DNA-DNA hybridization relatedness for isolates to be assigned to the same species (51), six different OTUs could be recognized. This observation suggests that the currently recognized subgroups of *C. trachomatis* and *C. psittaci* are more distinct from each other than they appear to be based on sequence data from only a minor part of the genome. Although these different groups could also be recognized by AFLP, the clustering pattern of the groups as calculated from AFLP fingerprints was different from that calculated from DNA-DNA hybridization data.

Analysis by AFLP has several advantages over other genome-based methods for typing *Chlamydia* isolates. In RFLP analysis, many different restriction enzymes and combinations had to be used to achieve the same level of discrimination (1, 5, 9, 27, 28, 37, 38, 42). RAPD assays are very difficult to standardize compared to AFLP analysis, since the PCR conditions for RAPD are of low stringency and therefore prone to variation (50) whereas the PCR conditions in AFLP analysis are of high stringency. Furthermore, RFLP analysis of genomic DNA requires large amounts of DNA (1 to 2 μg), or radioactively labeled DNA, to visualize the generated fragments. Therefore, the major advantage of the AFLP method is that it requires only one restriction enzyme combination and much less genomic DNA (<10 ng).

In conclusion, by using AFLP analysis of genomic DNA, differences among the four currently recognized *Chlamydia* species, and also within species, were observed. Furthermore, the cluster analysis of the AFLP fingerprints provides suggestions for a grouping of chlamydiae based on the whole genome. In addition, the genome of *C. pneumoniae* appeared to be highly conserved between isolates of respiratory and atherosclerotic origin as well.

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