Phylogenetic Analysis of the Genus *Chlamydia* Based on 16S rRNA Gene Sequences

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The phylogenetic relationships among *Chlamydia* spp. were investigated by comparing 16S rRNA gene sequences. In this analysis we used 14 strains of *Chlamydia psittaci*, including seven feline isolates, two avian isolates, two human isolates, one bovine isolate, one ovine isolate, and one guinea pig isolate; five strains of *Chlamydia pecorum*, including three bovine isolates, one ovine isolate, and one koala isolate; and nine strains of *Chlamydia trachomatis*, including six human isolates, two swine isolates, and one mouse isolate. A phylogenetic analysis of the 16S rRNA gene sequences of these organisms and seven previously published sequences revealed eight genetic groups which formed two clusters. The first cluster was composed of *C. pecorum*, *Chlamydia pneumoniae*, and *C. psittaci* and included three genetic groups (one group containing avian, human, and ovine strains, one group containing feline strains, and one group containing guinea pig strains). The second cluster was composed of *C. trachomatis* and also included three genetic groups (one group containing human strains, one group containing swine isolates, and one group containing rodent strains). The strains in each genetic group exhibited similar genetic distances. The results of the phylogenetic analysis agreed with the results of previous genomic DNA, *ompA* gene allele, and biotyping studies. Therefore, the genetic groups based on genetic distances may be considered a criterion for species identification.

Chlamydiae are obligatory intracellular bacteria that have a unique developmental cycle (25). These organisms are currently placed in the order Chlamydiales, family Chlamydiaceae, and genus *Chlamydia*. The following four species have been recognized in the genus: *Chlamydia trachomatis*, *Chlamydia psittaci*, *Chlamydia pneumoniae*, and *Chlamydia pecorum* (6, 14, 25). Differentiation of these species is based on the following criteria: inclusion morphology, the presence of glycogen in inclusions, sulfadiazine sensitivity, and DNA-DNA homology values (6, 13, 14, 26). *C. trachomatis*, the type species of the genus *Chlamydia*, and *C. psittaci* were the first two species assigned to the genus and were distinguished on the basis of differences in inclusion morphology, glycogen content, and susceptibility to sulfadiazine (13, 26). *C. pneumoniae* was initially identified as *C. psittaci*, because its phenotypic characteristics are similar to those of *C. psittaci* (i.e., dense inclusions, glycogen negative, resistant to sulfadiazine). *C. pneumoniae* was, however, proposed as a new species on the basis of ultrastructural differences in the morphology of its elementary bodies, its levels of DNA-DNA homology with other chlamydiae, and its serological reactivity (14). *C. pecorum*, the fourth species, was also placed in *C. psittaci* on the basis of its phenotypic characteristics and was separated from *C. psittaci* on the basis of DNA-DNA homology values and immunological specificities (6, 7).

C. psittaci contains a variety of chlamydiae with different genetic, serological, and host-tropic properties. Our previous DNA-DNA hybridization analysis revealed levels of homology ranging from 14 to 95% between *C. psittaci* strains (5). The levels of DNA homology between mammalian strains and avian strains of *C. psittaci* were less than 70%; the mammalian strains could be separated into two groups, feline strains and

ovine abortion strains, and the ovine abortion strains were more closely related to the avian strains than the feline strains were. The results of a molecular analysis of the nucleotide sequences of the *ompA* gene (23) were consistent with the results of the DNA-DNA homology analyses. Our previous report indicated that restriction fragment length polymorphism (RFLP) of the rRNA operon region is a useful marker for typing and species identification (8).

Recently, the classification of bacterial species has been re-evaluated by using genetic methods, including DNA-DNA homology, rRNA-DNA homology, and comparisons of 5S, 16S, and 23S rRNA sequences, as well as other biochemical methods, including multilocus enzyme electrophoresis and fatty acid composition analysis (24, 29–31, 36). Among the molecular analysis methods, rRNA or ribosomal DNA (rDNA) sequence comparisons provide a useful approach for studying phylogeny, especially the phylogeny of microorganisms with similar phenotypes, like the chlamydiae. rRNA gene sequences of avian *C. psittaci* (34), *C. trachomatis* (34), and *C. pneumoniae* (9, 10, 34) strains have been reported. However, the sequences of *C. pecorum* and most of the mammal-derived *C. psittaci* strains have not been analyzed previously.

In this paper, we describe an analysis of the 16S rRNA gene sequences of *C. psittaci*, *C. pecorum*, and *C. trachomatis* strains. We also reviewed the sequences of *C. pneumoniae* and *C. trachomatis* to investigate the phylogenetic relationships of these organisms in the genus *Chlamydia*. Our results indicated that the members of the genus *Chlamydia* can be separated into eight genetically distinguishable groups which form two genetic clusters.

MATERIALS AND METHODS

Chlamydial strains and DNAs. The chlamydial strains used in this study were 14 strains of *C. psittaci*, five strains of *C. pecorum*, and nine strains of *C. trachomatis* (Table 1). The procedures used for preparation of chlamydial DNA have been described previously (4). Koala type II DNA was kindly provided by N. Itoh, Yokohama City University, Yokohama, Japan. *C. trachomatis* A/Har-13^T was kindly provided by C.-C. Kuo, University of Washington, Seattle.

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TABLE 1. Chlamydial strains investigated

Species	Strain	Host	Geographical location	Disease	RFLP type ^a	Nucleotide sequence accession no.
<i>C. psittaci</i>	Prt/GCP-1	Parrot	Japan	Systemic infection	Av1a	D85713
	Frt-Hu/Cal10	Ferret, human	United States	Meningopneumonitis	Av1b	D85712
	Prk/6BC ^T (= ATCC VR125 ^T)	Parakeet	United States	Systemic infection	Av1c	M13769 ^b
	Hu/Borg (= ATCC VR601)	Human	United States	Psittacosis	Av1c	D85711
	Prk/Daruma	Parakeet	Japan (India)	Systemic infection	Av2a	D85710
	Ov/B577 (= ATCC VR656)	Sheep	United States	Abortion	Av2b	D85709
	EBA	Cattle	United States	Abortion		U73108
	CPEAE	Sheep	United Kingdom	Abortion		Z49871 ^b
	CPOAE	Sheep	United Kingdom	Abortion		Z49872 ^b
	Gp/Ic (= ATCC VR813)	Guinea pig	United States	Conjunctivitis	Gp1	D85708
	Fe/Pn-1 (= ATCC VR120)	Cat	United States	Pneumonia	Fe1a	D85701
	Fe/145	Cat	United States	Conjunctivitis	Fe1a	D85702
	Fe/Cello	Cat	United States	Conjunctivitis	Fe1b	D85706
	Fe/B166	Cat	United Kingdom	Conjunctivitis	Fe1b	D85703
	Fe/C164	Cat	United Kingdom	Conjunctivitis	Fe1b	D85704
	Fe/C429	Cat	United Kingdom	Conjunctivitis	Fe1b	D85707
	Fe/C454	Cat	United Kingdom	Conjunctivitis	Fe1b	D85705
<i>C. pecorum</i>	Bo/E58 ^T (= ATCC VR628 ^T)	Cattle	United States	Encephalomyelitis	Ru1a	D88317
	Bo/Maeda	Cattle	Japan	Pneumonia	Ru1a	D85715
	Bo/Shizuoka	Cattle	Japan	Encephalomyelitis	Ru1a	D85714
	Ov/IPA (= ATCC VR629)	Sheep	United States	Polyarthritis	Ru1b	D85716
	Koala (type II)	Koala	Australia	Urogenital infection		D85717
<i>C. pneumoniae</i>	TW183 ^T	Human	Taiwan	Conjunctivitis		L06108 ^b
	IOL207	Human	United Kingdom	Pneumonia		Z49874 ^b
<i>C. trachomatis</i>	A/Har-13 ^T	Human	Egypt	Trachoma		D89067
	A/SA-1/OT	Human	South Africa	Trachoma		D88316
	B/TW-5/OT	Human	Taiwan	Trachoma		D85719
	C/TW-3/OT	Human	Taiwan	Trachoma		D85720
	D/UW-3/Cx	Human	United States	Cervitis		D85721
	E/UW-5/Cx	Human	United States	Cervitis	CtHu	D85722
	L2/434/Bu	Human	United States	Lymphogranuloma venereum	CtHu	M59178 ^b
	R22	Pig	United States	Conjunctivitis		U73109
	S45	Pig	Austria	Asymptomatic		U73110
	MoPn/Weiss-Nigg	Mouse	United States	Pneumonia	CtMo	D85718
	SFPD	Hamster	United States	Proliferative ileitis		M83313 ^b

^a RFLP types were determined previously (5).

^b Previously published 16S rRNA sequence.

PCR amplification of chlamydial 16S rRNA gene fragments. Chlamydial 16S rRNA gene fragments were amplified by using a pair of primers (Table 2) constructed from the 16S rRNA gene sequence of *C. psittaci* Prk/6BC^T (T = type strain) (34). These primers contain restriction sites for *Bam*HI and *Sal*I, which is convenient for further cloning. Amplification was performed in 100-μl mixtures which contained 1 to 5 ng of genomic DNA and each primer at a concentration of 0.2 μM in 10 mM Tris-HCl buffer (pH 8.3) containing 50 mM KCl, 1.5 mM

MgCl₂, and each deoxynucleoside triphosphate at a concentration of 200 μM. The reactants were overlaid with 50 μl of mineral oil and subjected to thermal cycle amplification. The program consisted of initial melting at 94°C for 3 min, four cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min, 36 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, and a final extension step at 72°C for 10 min, followed by soaking at 4°C; a model 9600 GeneAmp PCR System thermal cycler (Perkin-Elmer Cetus, ABI, Tokyo, Japan) was used. Amplified products were purified by agarose gel electrophoresis. DNA was extracted from agarose gels by using a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.) as recommended by the manufacturer.

Sequencing of rDNA. The 16S rRNA gene fragment was inserted into pTV119N between the *Sal*I and *Bam*HI sites and then transfected into *Escherichia coli* MV1184. Recombinant plasmids were extracted and purified by the alkaline method (3). Direct sequences were also examined by using purified PCR products. Nucleotide sequences were determined by the dideoxy chain termination method (28). All sequencing was performed with AutoRead and AutoCycle sequencing kits and an ALFred sequencer (model 373A automated DNA sequencing system; Pharmacia, Tokyo, Japan). The sequencing primers used were -40 forward and reverse primers for pTV119N sites and internal primers for sites within the 16S rRNA gene (Table 2). Sequences were determined for both strands at least twice and were edited with the GCG Wisconsin computer program package (1).

Sequence analysis. In addition to the 16S rRNA gene sequences determined in this study, previously published sequences were also analyzed. These included the sequences of *C. psittaci* Prk/6BC^T (accession no. M13769), CPEAE (accession no. Z49871), and CPOAE (accession no. Z49872), *C. pneumoniae* TW183^T (accession no. L06108) and IOL207 (accession no. Z49874), *C. trachomatis* SFPD (accession no. M83313) and L2/434/Bu (accession no. M59178), and the

TABLE 2. Primers used for rDNA amplification and direct sequencing

Primer	Positions ^a	Sequence
16S1	-2 to 18	5'-CGGATCCTGAGAATTTGATC-3'
16S2	1554 to 1537	5'-TGTCGACAAAGGAGGTGATCCA-3'
rRNA1a	-1 to 21	5'-GGATCCTGAGAATTTGATCTTG-3'
rRNA1b	547 to 523	5'-TCCGTATTACCGCAGCTGCTGGCAC-3'
rRNA01	523 to 547	5'-GTGCCAGCAGCTGCGTAATACGGA-3'
rRNA02	742 to 718	5'-GCCTTCGCCACTGGTGTCTTCCAC-3'
rRNA03	886 to 912	5'-TATGCCGCTGAGGAGTACACTCGC-3'
rRNA04	1076 to 1052	5'-TGACGACAGCCATGCAGCACTGTG-3'
rRNA4b	1077 to 1099	5'-GCTCGTGCCGTGAGGTGTTGGGTTA-3'
rRNA4a	1554 to 1534	5'-TGTCGACAAAGGAGGTGATCCAGCC-3'

^a The positions of nucleotides were determined by using an alignment of the 16S rRNA gene sequences of 35 strains of chlamydiae.

Variable region	I		II		III		IV		V	
	67	85	121	128	172	197	224	244	462	480
Borg	-t-----c-----a--a				-a-.-		-g-----		ga-----c	
GCP-1	-t-----c-----a--a				-a-.-		-g-----		ga-----c	
Cal10	-t-----c-----a--a				-a-.-	-g--t-	-g-----		ga-----c	
6BC ⁷	-t-----c-----a--a				-a-.-	-t-	-g-----		ga-----c	
Daruma	-t-----c-----a--a				-a-.-	-t-			ga-----c	
B577	-t-----c-----a--a				-a-.-	-t-			ga-----c	
CPEAE	-t-----c-----a--a				-a-.-	-t-			ga-----c	
CPOAE	-t-----c-----a--a				-a-.-	-t-			ga-----c	
EBA	-t-----c-----a--a				-a-.-	-t-			ga-----c	
GPIC	-t--ag-g--g-a-a				-a-.-	-c--c--t-	-a-----			-c-
Fe/Pn1	-c-----g	-c-			-a-----		gg-a-----			-c-
Fe/145	-c-----g	-c-			-a-----		gg-a-----			-c-
Cello	-c-----g	-c-			-a-----		gg-a-----			-c-
C164	-c-----g	-c-			-a-----		gg-a-----			-c-
B166	-c-----g	-c-			-a-----		gg-a-----			-c-
C454	-c-----g	-c-			-a-----		gg-a-----			-c-
C429	-c-----g	-c-			-a-----		gg-a-----			-c-
E58 ⁷	-t---g---c-a-a	t---t-				-tt-	-a-a-g--		ga-----	
Maeda	-t---g---c-a-a	t---t-				-tt-	-a-a-g--		ga-----	
Shizuoka	-t---g---c-a-a	t---t-				-tt-	-a-a-g--		ga-----	
IPA	-t---g---c-a-a	t---t-				-tt-	-a-a-g--		ga-----	
Koala-II	-t---g---c-a-a	tgt--t-			-c--tt-		-a-a-g--		ga-----	
TW183 ⁷	-t-----a	-c-			a---aa-----t-t-t-		-g-a--t--c		ga-----c	
IOL207	-t-----a	-c-			a---aa-----t-t-t-		-g-a--t--c		ga-----c	
B	gc---tgt---acga--g	t-----t			-c-a-a--g---cg-gta-g				-ac--t---c-gc-g	
C	gc---tgt---acga--g	t-----t			-c-a-a--g---cg-gta-g				-ac--t---c-gc-g	
A ⁷	gc---tgt---acga--g	t-----t			-c-a-a--g---cg-gta-g				-ac--t---c-gc-g	
D	gc---tgt---acga--g	t-----t			-c-a-a--g---cg-gta-g				-ac--t---c-gc-g	
E	gc---tgt---caa--g	t-----t			-c-a-a--g---cg-gta-g				-ac--t---c-gc-g	
L2	gc---tgt---caa--g	t-----t			-c-a-a--g---cg-gta-g				-gc--t---c-gc-g	
R22	-c-g-t-.---aac-g	t---t-t			-cacca-----g-tgggtg		-a--g-----t		-gc--t---c-gcg	
S45	-cgg-t-.---a--a-c-g	t---t-t			-c-c-a-----g-tgggtg		-a--g-----t		-gc--t---c-gtcg	
MoPn	-c---tg-----ga--g	t-----t			-c-a-a-----tt-g		-a-----t		-ac--t---c-gt-g	
SFPD	-cg--tg-----ga--g	t-----t			-c-a-a-----tt-g		-a-----t		-ac--t---c-gt-g	
Consensus	A-AATGACTTCGGTTGTT-	CTGTCCCTC			GGTGTGTTTAGGCATCTAAAACACA		CGGTTAAGGGAGAGTCTATGG		AGTTGGCTAATATCCAATT	

Variable region	VI		VII		VIII	
	607	646	1005	1047	1455	1467
Borg	g--t-----c-a-----				-t-.-	
GCP-1	---t-----c-a-----				-.-	
Cal10	---t-----c-a-----				-.-	
6BC ⁷	---t-----c-a-----				-.-	
Daruma	---t-----c-a-----				-.-	
B577	---t-----c-a-----				-.-	
CPEAE	---t-----c-a-----				-.-	
CPOAE	---t-----c-a-----				-.-	
EBA	---t-----c-a-----				-.-	
GPIC	---t-----c-a-----				-.-	
Fe/Pn1			c-----		-.-	
Fe/145					-.-	
Cello					-.-	
C164					-.-	
B166					-.-	
C454					-.-	
C429					-.-	
E58 ⁷	---a-----t-----			-t-----	-at--tt---g	
Maeda	---a-----t-----				-at--tt---g	
Shizuoka	---a-----t-----				-at--tt---g	
IPA	---a-----t-----			-t-----	-at--tt---g	
Koala-II	---a-----t-----			-t-----	-at--tt---g	
TW183 ⁷	---t---t-----a---t---a		---aa-t-t---aca-c-----		-at--t-----	
IOL207	---t---t-----a---t---a		---aa-t-t---aca-c-----		-at--t-----	
B	t-----ga-c-----g		-a-----a-----t		c---g-.-	
C	t-----ga-c-----g		-a-----a-----t		c---g-.-	
A ⁷	t-----ga-c-----g		-a-----a-----t		c---g-.-	
D	t-----ga-c-----g		-a-----a-----t		c---g-.-	
E	t-----ga-c-----g		-a-----a-----t		c---g-.-	
L2	t-----ga-c-----g		-a-----a-----t		c---g-.-	
R22	t-----c-----g		c-a-----t-g		c---g-.-	
S45	t-----c-----g		c-a-----t-g		c---g-.-	
MoPn	t-----c-----g		c-a-----t-g		c---g-.-	
SFPD	t-----c-----g		c-a-----t-g		c---g-.-	
Consensus	ATGTCAAATCTTGGGGCTCAACCCCAAGTCGGCATCTAAT		TATTTGACCGCGCAGAAATGTCGTTTCCGCAAGGACAGATA		TGCTTAAAGGAGA	

FIG. 1. Comparison of the variable regions of the 16S rRNA gene sequences of *C. psittaci*, *C. pecorum*, *C. pneumoniae*, and *C. trachomatis*. Consensus sequences are shown at the bottom. Nucleotides that are different from the nucleotides in the consensus sequence are in lowercase letters. Dots indicate deletions.

TABLE 3. Dissimilarities and genetic distances in rDNA sequences of *Chlamydia* spp.

Strain	Dissimilarity or genetic distance ^a																
	Hu/ Borg	Prt/ GCP-1	Frt-Hu/ Cal10	Prk/ 6BC ^T	Prk/ Daruma	Ov/ B577	CPEAE	CPOAE	EBA	Gp/Ic	Fe/ Pn-1	Fe/ 145	Fe/ Cello	Fe/ C164	Fe/ B166	Fe/ C454	Fe/ C429
Hu/Borg	0	0.58	0.52	0.39	0.71	0.78	0.75	0.75	0.71	1.36	2.00	1.68	1.74	1.81	1.81	1.94	1.81
Prt/GCP-1	0.0058	0	0.32	0.19	0.52	0.58	0.55	0.55	0.52	1.29	1.81	1.49	1.55	1.61	1.61	1.74	1.55
Frt-Hu/Cal10	0.0052	0.0032	0	0.13	0.45	0.52	0.48	0.48	0.45	1.16	1.74	1.42	1.49	1.55	1.55	1.68	1.55
Prk/6BC ^T	0.0039	0.0019	0.0013	0	0.32	0.39	0.34	0.34	0.32	1.1	1.61	1.29	1.36	1.42	1.42	1.55	1.42
Prk/Daruma	0.0071	0.0052	0.0045	0.0032	0	0.32	0.27	0.27	0.26	1.03	1.94	1.61	1.68	1.74	1.74	1.87	1.74
Ov/B577	0.0078	0.0058	0.0052	0.0039	0.0032	0	0.07	0.07	0.06	1.10	2.00	1.68	1.74	1.81	1.81	1.94	1.81
CPEAE	0.0075	0.0055	0.0048	0.0034	0.0027	0.0007	0	0	0	1.09	2.05	1.71	1.77	1.84	1.84	1.98	1.84
CPOAE	0.0075	0.0055	0.0048	0.0034	0.0027	0.0007	0.0000	0	0	1.09	2.05	1.71	1.77	1.84	1.84	1.98	1.84
EBA	0.0071	0.0052	0.0045	0.0032	0.0026	0.0006	0.0000	0.0000	0	1.03	1.94	1.61	1.68	1.74	1.74	1.87	1.74
Gp/Ic	0.0137	0.0130	0.0117	0.0111	0.0104	0.0111	0.0110	0.0110	0.0104	0	2.07	1.74	1.81	1.87	1.87	2.00	1.87
Fe/Pn-1	0.0203	0.0183	0.0176	0.0163	0.0196	0.0203	0.0207	0.0207	0.0196	0.0210	0	0.32	0.39	0.32	0.45	0.52	0.45
Fe/145	0.017	0.0150	0.0143	0.0130	0.0163	0.0170	0.0173	0.0173	0.0163	0.0176	0.0032	0	0.06	0.13	0.13	0.26	0.13
Fe/Cello	0.0176	0.0157	0.0150	0.0137	0.0170	0.0176	0.0179	0.0179	0.0170	0.0183	0.0039	0.0006	0	0.06	0.19	0.19	0.06
Fe/C164	0.0183	0.0163	0.0157	0.0143	0.0176	0.0183	0.0186	0.0186	0.0176	0.0190	0.0032	0.0013	0.0006	0	0.26	0.19	0.13
Fe/B166	0.0183	0.0163	0.0157	0.0143	0.0176	0.0183	0.0186	0.0186	0.0176	0.0190	0.0045	0.0013	0.0019	0.0026	0	0.39	0.26
Fe/C454	0.0196	0.0176	0.0170	0.0157	0.0190	0.0196	0.0200	0.0200	0.0190	0.0203	0.0052	0.0026	0.0019	0.0019	0.0039	0	0.26
Fe/C429	0.0183	0.0157	0.0157	0.0143	0.0176	0.0183	0.0186	0.0186	0.0176	0.0190	0.0045	0.0013	0.0006	0.0013	0.0026	0.0026	0
Bo/E58 ^T	0.0364	0.0344	0.0358	0.0351	0.0371	0.0378	0.0393	0.0393	0.0371	0.0392	0.0426	0.0392	0.0399	0.0405	0.0405	0.0419	0.0405
Bo/Maeda	0.0371	0.0364	0.0378	0.0371	0.0392	0.0399	0.0414	0.0414	0.0392	0.0412	0.0446	0.0412	0.0419	0.0426	0.0426	0.0440	0.0426
Bo/Shizuoka	0.0371	0.0364	0.0378	0.0371	0.0392	0.0399	0.0414	0.0414	0.0392	0.0412	0.0446	0.0412	0.0419	0.0426	0.0426	0.0440	0.0426
Ov/IPA	0.0371	0.0351	0.0364	0.0358	0.0378	0.0385	0.0400	0.0400	0.0378	0.0399	0.0433	0.0399	0.0405	0.0412	0.0412	0.0426	0.0412
Koala-II	0.0371	0.0351	0.0364	0.0358	0.0378	0.0385	0.0400	0.0400	0.0378	0.0399	0.0433	0.0399	0.0405	0.0412	0.0412	0.0426	0.0412
TW183 ^T	0.0412	0.0384	0.0405	0.0391	0.0425	0.0432	0.0443	0.0443	0.0425	0.0487	0.0508	0.0473	0.0480	0.0487	0.0487	0.0501	0.0487
IOL207	0.0412	0.0384	0.0405	0.0391	0.0425	0.0432	0.0443	0.0443	0.0425	0.0487	0.0508	0.0473	0.0480	0.0487	0.0487	0.0501	0.0487
B/TW-5/OT	0.0522	0.0529	0.0515	0.0508	0.0515	0.0522	0.0538	0.0538	0.0515	0.0494	0.0550	0.0515	0.0522	0.0529	0.0515	0.0543	0.0529
C/TW-3/OT	0.0522	0.0529	0.0515	0.0508	0.0515	0.0522	0.0538	0.0538	0.0515	0.0494	0.0550	0.0515	0.0522	0.0529	0.0529	0.0543	0.0529
A/Har-13 ^T	0.0515	0.0522	0.0508	0.0501	0.0508	0.0515	0.0530	0.0530	0.0508	0.0487	0.0543	0.0508	0.0515	0.0522	0.0522	0.0536	0.0522
D/UW-3/Cx	0.0515	0.0522	0.0508	0.0501	0.0508	0.0515	0.0530	0.0530	0.0508	0.0487	0.0543	0.0508	0.0515	0.0522	0.0522	0.0536	0.0522
E/UW-5/Cx	0.0501	0.0508	0.0494	0.0487	0.0494	0.0515	0.0530	0.0530	0.0508	0.0487	0.0529	0.0494	0.0501	0.0508	0.0494	0.0522	0.0508
L2/434/Bu	0.0494	0.0501	0.0487	0.0480	0.0501	0.0508	0.0515	0.0515	0.0501	0.0480	0.0522	0.0487	0.0494	0.0501	0.0501	0.0515	0.0501
R22	0.0510	0.0517	0.0503	0.0496	0.0517	0.0524	0.0539	0.0539	0.0517	0.0510	0.0538	0.0503	0.0510	0.0517	0.0517	0.0531	0.0517
S45	0.0577	0.0570	0.0556	0.0549	0.0570	0.0577	0.0591	0.0591	0.0570	0.0577	0.0577	0.0542	0.0549	0.0556	0.0556	0.0570	0.0556
MoPn/Weiss- Nigg	0.0446	0.0453	0.0439	0.0432	0.0439	0.0446	0.0464	0.0464	0.0439	0.0439	0.0460	0.0425	0.0432	0.0439	0.0439	0.0453	0.0439
SFPD	0.0463	0.0471	0.0456	0.0449	0.0456	0.0463	0.0464	0.0464	0.0456	0.0456	0.0485	0.0449	0.0456	0.0463	0.0463	0.0478	0.0463
"Z"	0.1773	0.1765	0.1756	0.1748	0.1765	0.1773	0.1829	0.1829	0.1765	0.1724	0.1756	0.1716	0.1724	0.1732	0.1732	0.1748	0.1732

^a The values on the upper right are numbers of substitutions per 100 bases. The values on the lower left are the genetic distances calculated by the Jukes-Cantor method. The values inside each solid box are the values for a species. The values inside the dotted boxes are sequence dissimilarity values of less than 1% and genetic distances of less than 0.01. The rDNA sequence of strain SFPD contains 19 ambiguous sites, which were not included in the analysis.

chlamydia-like microorganism *Simkania* sp. strain "Z" (accession no. L27666); these sequences were obtained from the DNA Data Bank of Japan (DDBJ), Mishima, Japan. The 16S rRNA gene sequences of *C. psittaci* EBA (accession no. U73108) and *C. trachomatis* R22 (accession no. U73109) and S45 (accession no. U73110) were kindly provided by Karin D. E. Everett and Arthur A. Andersen of the Avian and Swine Respiratory Diseases Research Unit, USDA Agricultural Research Service National Animal Disease Center, Ames, Iowa. Multiple alignments of the 16S rRNA gene sequences were examined by using the computer program ClustalW (17) and a SUN workstation. Genetic distances were calculated by the method of Jukes and Cantor (21). A phylogenetic tree was constructed by the neighbor-joining method (27). The molecular phylogenetic analysis results were analyzed by using the program package ODEN (20) at the National Institute of Genetics, Mishima, Japan, the PHYLIP package (2), and the GCG Wisconsin package (1) on the SUN workstation at Gifu University Computing Center, Gifu, Japan.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers listed in Table 1.

RESULTS

The 16S rRNA genes of the *Chlamydia* strains analyzed were amplified with primers 16S1 and 16S2. The following two strategies were employed to determine the sequences: (i) cloning-subcloning and sequencing and (ii) direct sequencing. The

cloning-subcloning method was initially used for the PCR products from *C. psittaci* Prt/GCP-1, Prk/Daruma, and Fe/Pn-1 and *C. pecorum* Bo/Maeda and Bo/Shizuoka. Based on these sequences and the previously reported sequences, internal direct sequencing primers were designed for the chlamydial 16S rRNA gene (Table 2) for subsequent direct sequencing. In order to confirm the fidelity of the direct sequencing, the sequence of the 16S rRNA gene of Prk/6BC^T was determined for comparison with the previously reported sequence (34). Only one nucleotide mismatch between the two sequences was found. This mismatch was reproducible in several repeated sequence analyses. Therefore, the mismatch was not caused by a sequencing error. Thus, the reliability of the sequences determined was greater than 99.94%.

The 16S rRNA gene sequences of 27 chlamydial strains were determined. In addition, seven sequences from DNA databases were included in the analysis. The 16S rRNA gene sequences were composed of 1,548 bp in both *C. psittaci* and human *C. trachomatis* strains, 1,544 bp in swine *C. trachomatis* strains, 1,549 bp in both rodent *C. trachomatis* and *C. pecorum* strains, and 1,550 bp in *C. pneumoniae* strains. The consensus

TABLE 3—Continued

Dissimilarity or genetic distance ^a																	
Bo/ E58 ^T	Bo/ Maeda	Bo/ Shizuoka	Ov/ IPA	Koala II	TW183 ^T	IOL207	B/ TW-5/ OT	C/ TW-3/ OT	A/ Har-13 ^T	D/ UW-3/ Cx	E/ UW-5/ Cx	L2/ 434/ Bu	R22	S45	MoPn/ Weiss- Nigg	SFPD	"Z"
3.56	3.62	3.62	3.62	3.62	4.01	4.01	5.04	5.04	4.98	4.98	4.85	4.78	4.93	5.56	4.33	5.71	15.8
3.36	3.56	3.56	3.43	3.43	3.75	3.75	5.11	5.11	5.04	5.04	4.91	4.84	4.99	5.49	4.39	5.78	15.7
3.49	3.69	3.69	3.56	3.56	3.94	3.94	4.98	4.98	4.91	4.91	4.78	4.72	4.86	5.36	4.26	5.65	15.7
3.43	3.62	3.62	3.49	3.49	3.81	3.81	4.91	4.91	4.85	4.85	4.72	4.65	4.8	5.29	4.2	5.58	15.6
3.62	3.82	3.82	3.69	3.69	4.13	4.13	4.98	4.98	4.91	4.91	4.78	4.84	4.99	5.49	4.26	5.65	15.7
3.69	3.88	3.88	3.75	3.75	4.20	4.20	5.04	5.04	4.98	4.98	4.98	4.91	5.06	5.56	4.33	5.71	15.8
3.83	4.03	4.03	3.89	3.89	4.30	4.30	5.19	5.19	5.12	5.12	5.12	4.98	5.20	5.68	4.50	5.74	16.2
3.83	4.03	4.03	3.89	3.89	4.30	4.30	5.19	5.19	5.12	5.12	5.12	4.98	5.20	5.68	4.50	5.74	16.2
3.62	3.82	3.82	3.69	3.69	4.13	4.13	4.98	4.98	4.91	4.91	4.91	4.84	4.99	5.49	4.26	5.65	15.7
3.82	4.01	4.01	3.88	3.88	4.72	4.72	4.78	4.78	4.72	4.72	4.72	4.65	4.93	5.56	4.26	5.65	15.4
4.14	4.33	4.33	4.20	4.20	4.91	4.91	5.30	5.30	5.24	5.24	5.11	5.04	5.19	5.56	4.46	5.91	15.7
3.82	4.01	4.01	3.88	3.88	4.59	4.59	4.98	4.98	4.91	4.91	4.78	4.72	4.86	5.23	4.13	5.58	15.3
3.88	4.08	4.08	3.95	3.95	4.65	4.65	5.04	5.04	4.98	4.98	4.85	4.78	4.93	5.29	4.20	5.65	15.4
3.95	4.14	4.14	4.01	4.01	4.72	4.72	5.11	5.11	5.04	5.04	4.91	4.84	4.99	5.36	4.26	5.71	15.5
3.95	4.14	4.14	4.01	4.01	4.72	4.72	4.98	5.11	5.04	5.04	4.78	4.84	4.99	5.36	4.26	5.71	15.5
4.08	4.27	4.27	4.14	4.14	4.84	4.84	5.24	5.24	5.17	5.17	5.04	4.97	5.12	5.49	4.39	5.85	15.6
3.95	4.14	4.14	4.01	4.01	4.72	4.72	5.11	5.11	5.04	5.04	4.91	4.84	4.99	5.36	4.26	5.71	15.5
0	0.32	0.32	0.06	0.32	4.20	4.20	4.98	4.98	4.92	4.92	4.85	4.79	5.26	5.43	4.40	5.79	15.7
0.0032	0	0.00	0.26	0.65	4.39	4.39	5.18	5.18	5.11	5.11	5.05	4.98	5.45	5.63	4.59	5.99	15.8
0.0032	0.0000	0	0.26	0.65	4.39	4.39	5.18	5.18	5.11	5.11	5.05	4.98	5.45	5.63	4.59	5.99	15.8
0.0006	0.0026	0.0026	0	0.39	4.26	4.26	5.05	5.05	4.98	4.98	4.92	4.85	5.32	5.5	4.46	5.85	15.8
0.0032	0.0065	0.0065	0.0039	0	4.39	4.39	4.98	4.98	4.92	4.92	4.85	4.79	5.32	5.5	4.46	5.85	15.7
0.0432	0.0453	0.0453	0.0439	0.0453	0	0.00	6.14	6.14	6.08	6.08	6.08	6.01	6.49	6.47	5.49	6.92	15.9
0.0432	0.0453	0.0453	0.0439	0.0453	0.0000	0	6.14	6.14	6.08	6.08	6.08	6.01	6.49	6.47	5.49	6.92	15.9
0.0516	0.0537	0.0537	0.0523	0.0516	0.0641	0.0641	0	0.13	0.06	0.06	0.39	0.52	2.59	2.68	1.68	3.02	15.8
0.0516	0.0537	0.0537	0.0523	0.0516	0.0641	0.0641	0.0013	0	0.06	0.06	0.52	0.52	2.59	2.68	1.68	3.02	15.8
0.0509	0.0530	0.0530	0.0516	0.0509	0.0634	0.0634	0.0006	0.0006	0	0	0.45	0.45	2.53	2.61	1.61	2.96	15.7
0.0509	0.0530	0.0530	0.0516	0.0509	0.0634	0.0634	0.0006	0.0006	0.0000	0	0.45	0.45	2.53	2.61	1.61	2.96	15.7
0.0502	0.0523	0.0523	0.0509	0.0502	0.0634	0.0634	0.0039	0.0052	0.0045	0.0045	0	0.26	2.4	2.48	1.68	3.02	15.7
0.0495	0.0515	0.0515	0.0502	0.0495	0.0626	0.0626	0.0052	0.0052	0.0045	0.0045	0.0026	0	2.27	2.35	1.68	2.96	15.5
0.0545	0.0566	0.0566	0.0552	0.0552	0.0678	0.0678	0.0264	0.0264	0.0257	0.0257	0.0244	0.0230	0	1.05	2.01	3.37	15.1
0.0564	0.0585	0.0585	0.0571	0.0571	0.0677	0.0677	0.0273	0.0273	0.0266	0.0266	0.0253	0.0239	0.0105	0	2.16	3.33	15.4
0.0453	0.0474	0.0474	0.0460	0.0460	0.0570	0.0570	0.0170	0.0170	0.0163	0.0163	0.0170	0.0170	0.0204	0.0219	0	1.54	15.5
0.0471	0.0493	0.0493	0.0478	0.0478	0.0595	0.0595	0.0179	0.0179	0.0172	0.0172	0.0179	0.0172	0.0215	0.0210	0.0027	0	17.0
0.1767	0.1776	0.1776	0.1776	0.1759	0.1781	0.1781	0.1773	0.1773	0.1765	0.1765	0.1756	0.1739	0.1685	0.1724	0.1731	0.1793	0

sequence was constructed and assigned a number based on the multiple alignment. Nucleotide insertions or deletions were observed at 12 sites, including positions 73, 74, 77, 78, 79, 128, 178, 753, 1,458, 1,459, 1,464, and 1,465. Variations in the chlamydial 16S rRNA gene sequences were evident in eight areas (Fig. 1). These areas were designated variable regions I to VIII, which corresponded to nucleotides 67 to 85, 121 to 128, 172 to 197, 224 to 244, 462 to 480, 607 to 646, 1005 to 1047, and 1455 to 1467, respectively. These regions were mapped to the double helical stem or hairpin portions of the predicted secondary structure of the 16S rRNA molecule (16). The sequences in these variable regions exhibited genetic group specificity.

The sequences of the chlamydial 16S rRNA gene were highly conserved. The levels of sequence dissimilarity and the genetic distances were 0 to 6.21% and 0 to 0.0648, respectively, among the chlamydiae (Table 3). The previously published sequences of *C. pneumoniae* TW183^T and IOL207, which were obtained from the DDBJ, were identical. The distribution of dissimilarities and genetic distances revealed three peaks. The levels of dissimilarity for these peaks ranged from 0 to 0.78%, from 1.09 to 2.10%, and from 3.43 to 6.21%, and the genetic distances ranged from 0 to 0.0078, from 0.0104 to 0.0210, and

from 0.0351 to 0.0648. The levels of dissimilarity and genetic distances within species were 0 to 2.07% and 0 to 0.0210, respectively, in *C. psittaci*, 0 to 0.65% and 0 to 0.0065, respectively, in *C. pecorum*, and 0 to 3.37% and 0 to 0.0273, respectively in *C. trachomatis*. Thus, the *C. psittaci* and *C. trachomatis* sequence data revealed two peaks of dissimilarity and genetic distance within the species. Sequences that fell within the range of values for each peak were considered members of a group. A total of eight groups were identified. These groups consisted of (i) avian and ovine abortion strains of *C. psittaci*, (ii) a guinea pig strain of *C. psittaci*, (iii) feline strains of *C. psittaci*, (iv) *C. pecorum*, (v) *C. pneumoniae*, (vi) human strains of *C. trachomatis*, (vii) swine strains, and (viii) rodent strains of *C. trachomatis*.

A phylogenetic tree was constructed from the genetic distances determined by the neighbor-joining method (Fig. 2). This tree contained two major clusters of chlamydial strains. The first cluster was composed of five subclusters (avian and ovine bovine abortion strains of *C. psittaci*, guinea pig strains of *C. psittaci*, feline strains of *C. psittaci*, *C. pecorum*, and *C. pneumoniae*). The second cluster was composed of three subclusters (human strains, swine strains, and rodent strains of *C. trachomatis*). These subclusters corresponded to the genetic

FIG. 2. Phylogenetic tree for *Chlamydia* spp. based on a sequence analysis of the 16S rRNA gene. The tree was constructed by the neighbor-joining method from phylogenetic distances calculated by the Jukes-Cantor method (Table 3). The tree root is at the midpoint. The numbers at the branch points are bootstrap values based on 1,000 replicates. Horizontal distances correspond to genetic distances; vertical distances are arbitrary.

groups described above. A bootstrap evaluation was performed to determine the confidence level for the phylogenetic tree.

The sequence of the chlamydia-like organism *Simkania* sp. strain "Z" was also compared with the sequences of chlamydiae. This sequence exhibited 15.40 to 17.0% dissimilarity and genetic distances of 0.1685 to 0.1829 compared with the chlamydiae.

DISCUSSION

A phylogenetic analysis of 16S rRNA gene sequences revealed eight groups that formed two clusters within the *Chlamydia* spp. Each cluster corresponded to a phenotype defined by inclusion morphology, glycogen content of inclusions, sulfadiazine sensitivity, and number of rRNA gene loci. One of the clusters corresponded to *C. trachomatis*, which forms vacuolar inclusions, contains glycogen in its inclusions, is sulfadiazine sensitive, and has two rRNA operon loci. The other cluster included *C. psittaci*, *C. pecorum*, and *C. pneumoniae*, which form compact inclusions, contain no glycogen, are sulfadiazine resistant, and have only one rRNA operon locus. The 16S rRNA gene sequences of two strains of *C. pneumoniae* were identical, and the 16S rRNA gene sequences of *C. pecorum* strains were similar. Therefore, the strains of *C. pneumoniae* formed one genetic group, as did the strains of *C. pecorum*. However, strains of both *C. psittaci* and *C. trachomatis* fell into three genetic groups.

Our phylogenetic analysis provided a basis for further division of *C. psittaci* into several species. The members of each

genetic group of *C. psittaci* had similar biological properties, such as host range, pathogenesis, and biotypes as proposed by Spears and Storz (32). Furthermore, the genetic distances among the *C. psittaci* strains within each genetic group were almost identical to the genetic distances among *C. trachomatis* strains within biovars and the genetic distances among *C. pecorum* strains. These results were also consistent with the previous classification based on biotyping and *ompA* allelic analysis data (23, 32). Therefore, the genetic groups identified in the present study may be considered species.

It is interesting that the abortion strains were more closely related to avian strains than to feline or guinea pig strains. The close relationship between abortion and avian strains was also revealed by a genomic RFLP analysis (5) and an *ompA* allelic analysis (23). This relationship suggests that the abortion strains might have been transmitted from birds to ruminants and adapted to the new hosts. This question may be answered by retrospective epidemiological studies.

Despite the heterogeneity in host origin and in the *ompA* gene (23), *C. pecorum* strains seemed to have homogeneous rRNA sequences. When a new species was proposed for *C. pecorum*, the strains came from ruminants, mainly cattle and sheep. At present, pigs and koala strains have been classified as *C. pecorum* strains. However, no species-specific phenotypic characteristics other than immunological specificity in immunoblotting have been identified. Therefore, the 16S rRNA gene sequence should be a useful marker for identifying *C. pecorum*.

The koala strain tested in the present study was found to belong to *C. pecorum*. Two types of koala strains have been recognized (12, 15). Type I strains are isolated from conjunctivae, and type II strains are isolated from uteri, vaginas, and recta (12). The two types have been differentiated by restriction enzyme digestion, gene probe analysis, and the presence or absence of a 7.4-kb plasmid (11, 19). Our results showed that our koala type II strain was a *C. pecorum* strain. This grouping is consistent with the results of biotyping by the method of Spears and Storz, which showed that a koala type II strain is a biotype 2 strain (15), as are the polyarthritides and sporadic bovine encephalomyelitis isolates of *C. pecorum*. Nucleotide sequence data for the 16S rRNA gene of type I koala strains are not available yet. However, the sequence of the *ompA* gene of the type I koala strain was similar to the *C. pneumoniae* sequence (23). Further analysis of the 16S rRNA gene sequences of koala strains may clarify the phylogenetic status of these organisms.

The two biovars of human *C. trachomatis* strains formed a single genetic group. The 16S rRNA gene sequence of *C. trachomatis* biovar trachoma was similar to that of *C. trachomatis* biovar lymphogranuloma. The results indicated that there is a close relationship between *C. trachomatis* biovar trachoma and *C. trachomatis* biovar lymphogranuloma. The genetic groups of *C. trachomatis* did not seem to correspond to biovar designations. The human *C. trachomatis* strains have two well-defined serological complexes, the B and C complexes (33). The sequence analysis did not differentiate between the serological complexes. On the other hand, the sequences of mouse biovar strains and swine biovar strains were different from the sequences of the human biovar strains. The mouse biovar strains, as well as the swine biovar strains, formed a single genetic group.

Many chlamydia-like organisms have been obtained from various vertebrates and invertebrates, including African clawed frogs and turtles (18, 35). The PCR amplification and direct sequencing method described in this study may be used to study the molecular relatedness of these chlamydia-like organ-

isms to chlamydial species. By using these methods the chlamydia-like organism *Simkania* sp. strain "Z" (22) was shown to be distantly related to the genus *Chlamydia*.

The variability within *C. psittaci* and *C. trachomatis* was great. However, the variability within genetic groups of the two species was as low as the variability in *C. pecorum* and *C. pneumoniae*. This suggests that the genetic groups within *C. psittaci* and *C. trachomatis* defined in this study may be considered individual species.

In conclusion, genetic grouping should be useful for future classification but should not be the only criterion. Additional studies are needed to consider whether the genetic groups are new species and to construction a classification scheme for the chlamydiae.

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