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FULL PAPER

Molecular evidence for genetic distinctions between Chlamydiaceae detected in Siamese crocodiles (*Crocodylus siamensis*) and known Chlamydiaceae species

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Abstract

Chlamydiosis, caused by Chlamydiaceae, is a zoonotic disease found in humans and several species of animals, including reptiles and amphibians. Although chlamydiosis in saltwater crocodiles has been previously reported in South Africa and Papua New Guinea, the reported strains have not been identified or confirmed. Therefore, the main aim of this study was to sequence and characterize Chamydiaceae isolated from Siamese crocodiles. Results showed the 16S ribosomal (r) RNA and the 16S/23S rRNA gene of the crocodile isolates were closely related to the genus *Chlamydophila* with matched identity greater than 98%. The phylogenetic tree constructed from the 16S/23S rRNA gene showed the crocodile cluster diverges far from *Cp. caviae* with a 100% bootstrap value. The tree based on the *ompA* gene loci distinguished the crocodile strains into genotypes I, II, and III. The present study is the first report on *Chlamydophila* detected in Siamese crocodiles that is genetically distinct from the known species of Chlamydiaceae.

Key Words: Chlamydiaceae, *C. siamensis*, Siamese crocodiles

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Introduction

The family Chlamydiaceae, which causes chlamydiosis in humans and several species of animals, is an obligate intracellular gram-negative bacterium. The bacterium has a unique cycle of replication that consists of an infectious elementary body and a noninfectious reticulate body. Chlamydiaceae is divided into two genera, *Chlamydia* and *Chlamydophila*, based on the DNA sequence of the 16S and/or 23S ribosomal (r) RNA genes[6,10]. Until the present research, the six known species of *Chlamydi*a were *Chlamydia* (*C.*) *muridarum*, *C. suis*, *C. trachomatis*, *C. ibidis*, *C. avium*, and *C. gallinacean*. *Chlamydophila* was composed of *Chlamydophila* (*Cp.*) *pneumoniae*, *Cp. psittaci*, *Cp. abortus*, *Cp. caviae*, *Cp. percorum*, and *Cp. felis*[5,13,18]. Several Chlamydiaceae species such as *Cp. pneumoniae*, *Cp. psittaci*, *Cp. abortus*, and *Cp. felis* are found in reptiles, including crocodiles, snakes, chelonians, and lizards. Infected reptiles show clinical signs of eye discharge, fibrinous pharyngitis, conjunctivitis, pneumonia, hepatitis, and granulomatous lesions in the heart, liver, lung, spleen, and small intestine[1,14]. Chlamydiosis has been previously reported in farmed hatchling Nile crocodiles (*Crocodylus niloticus*) in South Africa and Zimbabwe. The bacterium found in and isolated from the crocodiles was presumed to be *Cp. psittaci*[10]. Additionally, an outbreak of chlamydiosis occurred in hatchling and juvenile Indo-Pacific crocodiles (*C. porosus*) on a farm in Papua New Guinea. Infected crocodiles exhibited high mortality from hepatitis and exudative conjunctivitis[11]. However, species and genetic characteristics of Chlamydiaceae involved in case in crocodiles have not been identified. The aim of this study was to detect and characterize Chlamydiaceae in Siamese crocodiles (*C. siamensis*).

Materials and methods

Sample collection and genomic DNA extraction: During 2012 and 2013, 31 Siamese crocodile (*C. siamensis*) carcasses were received from 14 farms located in central Thailand. Clinical specimens from each crocodile were collected. Genomic DNA was extracted from the tissue samples using the DNeasy blood and tissue kit (Qiagen). The DNA samples were kept at −20°C until use.

Chlamydiaceae detection: Primer specific to the *ompA* gene was used for Chlamydiaceae detection in a semi-nested polymerase chain reaction (PCR) format[3]. Nucleotide sequences of primers are listed in table 1. In the first PCR, primers A and B produced a 260-bp fragment. The PCR mixture contained 2 µl of template DNA, 2.5 µl of 10× Mg^{+2} free buffer, 1.5 mM of Mg^{+2} solution, 1 mM of dNTPs, 2.5 units of i-*Taq* DNA polymerase (iNtRON), and 0.5 µM each of A and B primer. Sterile nuclease-free water was added up to increase the mixture to 25 µl. The PCR was performed under the conditions of 2 minutes at 94°C for initial denaturing, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, and was terminated at 72°C for 7 minutes. In the second PCR, primers B and C amplified a 165-bp fragment. The PCR mixture and PCR parameters were the same as those of the first PCR, with the exception of the use of Mg^{+2} in the final concentration of 3 mM.

Mycoplasmas, iridovirus, and pan-herpesviruses detection: The crocodile samples were also tested for mycoplasmas, iridovirus, and pan-herpesviruses. The nucleotide sequences of primers are listed in table 1. Primers Myco-F and Myco-R were used for mycoplasmas detection and generated a 510-bp PCR product[9]. Primers FV3-F and FV3-R were used for iridovirus amplification and produced a 530-bp PCR product. For pan herpesviruses, primers DFA, IL, and KGI were used in the first PCR and primers TGV and IYG were used in the second PCR and produced a 207-bp PCR product[16]. The components of the PCR reaction were similar those described above. The PCR reaction for mycoplasmas was

Table 1. Primers used in this study

Target	Primer name	Primer sequence
Chlamydiaceae	Primer A	5' CAG GAT ATC TTG TCT GGC TTT AA 3'
	Primer B	5' GCA AGG ATC GCA AGG ATC 3'
	Primer C	5' TTA GAG GTG AGT ATG AAA AAA CTC 3'
Mycoplasmas	Myco-F	5' CGC CTG AGT AGT ACG TWC GC 3'
	Myco-R	5' GCG GTG TGT ACA ARA CCC GA 3'
Iridovirus	FV3-F	5' GAC TTG GCC ACT TAT GAC 3'
	FV3-R	5' GTC TCT GGA GAA GAA GAA 3'
Pan herpesviruses	DFA	5' GAY TTY GCN AGY YTN TAY CC 3'
	IL	5' TCC TGG ACA AGC AGC ARN YSG CNM TNA A 3'
	KGI	5' GTC TTG CTC ACC AGN TCN ACN CCY TT 3'
	TGV	5' TGT AAC TCG GTG TAY GGN TTY ACN GGN GT 3'
	IYG	5' CAC AGA GTC CGT RTC NCC RTA DAT 3'
Sequencing primers for *ompA* gene	CTU	5'-ATG AAA AAA CTC TTG AAA TCG G-3'
	CTL	5' CAA GAT TTT CTA GAY TTC ATY TTG TT 3'
	OMP-F	5' TCT TTC ATT GAT TAA GCG TGC T 3'
Sequencing primers for 16S rRNA gene	Chlamydophila_16SF	5' GCG TGG ATG AGG CAT GCA A 3'
	Chlamydophila_16SR	5' GGA GGT GAT CCA GCC CCA 3'
Sequencing primers for 16S/23S rRNA gene	U16SF	5' GCA TGT GGT ITA ATT CGA TGC AAC GCG AAG A 3'
	U23SR	5' GAA TTT CGC TAC CTT AGG ACC GTT ATA GTT AC 3'

performed under the conditions of 2 minutes at 94°C for initial denaturing, followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 65°C, and 30 seconds at 72°C, and was terminated at 72°C for 7 minutes. For iridovirus detection, the annealing temperature was 52°C. For pan herpesviruses, annealing temperatures of 50°C and 56°C were used in the first and second PCRs, respectively.

Phylogenetic markers amplification and DNA sequencing: The positive samples from diagnostic nested PCR were used for amplification of the phylogenetic markers *ompA*, 16S rRNA, and 16S/23S rRNA[4,5,6]. A 1,058-bp fragment of the *ompA* gene was amplified with primers CTU and CTL[4]. Where it was not possible to amplify the *ompA* gene by primers CTU and CTL, primers CTU and OMP-F were amplified to generate a 1,137-bp PCR product. A full length of 16S rRNA was amplified from chromosomal DNA using primers Chlamydophila_16SF and Chlamydophila_16SR and produced a PCR product of approximately 1,500-bp[5]. A portion of the ribosomal operon 16S/23S rRNA, composed of one-third of the 16S rRNA gene, an intergenic spacer, and the domain I segment of the 23S rRNA gene, was amplified by primers U16SF and U23SR and generated a PCR product size of approximately 2.8 kbps[6]. Twenty-five microliters of PCR reaction mixture contained 2 μl of template DNA, 2.5 μl of 10× Mg^{+2} free buffer, 1.5 mM of Mg^{+2} solution, 1 mM of dNTPs mix, 0.5 μl of i-*Taq* DNA polymerase (iNtRON), and 0.5 μM each of forward and reverse primer. For *ompA* gene amplification, the PCR was performed under the conditions of 2 minutes at 94°C for initial denaturing, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, and was terminated at 72°C for 7 minutes. For 16S and 16S/23S rRNA gene amplification, the PCRs were performed under the conditions of 2 minutes at 94°C for initial denaturing, followed by 35 cycles of 45 seconds at

Table 2. Result of Chlamydiaceae DNA detection by nested PCR in each organ of Siamese crocodiles

Year	Farm ID	Sample ID	Lung	Liver	Spleen	Brain
2012	5504	5504C01	+	+	+	+
2012	5508	5508C01	−	−	N.D.	N.D.
		5508C02	−	−	N.D.	N.D.
2012	5509	5509C05	+	+	N.D.	N.D.
		5509C06	N.D.	+	N.D.	N.D.
		5509C07	+	+	+	+
		5509C08	+	−	−	−
		5509C09	−	+	−	−
2012	5510	5510C01	+	+	N.D.	+
2012	5511	5511C01	−	−	N.D.	N.D.
		5511C02	+	+	+	N.D.
2012	5518	5518C01	+	+	+	+
2012	5520	5520C01	N.D.	+	N.D.	N.D.
2012	5522	5522C01	N.D.	+	N.D.	N.D.
		5522C02	−	−	−	−
2013	5602	5602C01	+	+	+	+
2013	5606	5606C01	+	N.D.	N.D.	N.D.
		5606C02	−	N.D.	N.D.	N.D.
2013	5607	5607C02	N.D.	+	+	+
2013	5609	5609C01/N	+	+	+	+
		5609C01/O	N.D.	+	N.D.	+
		5609C02/N	N.D.	+	N.D.	+
		5609C03/N	N.D.	+	N.D.	N.D.
2013	5620	5620C01	N.D.	−	−	−
		5620C02	+	+	+	+
2013	5632	5632C02	N.D.	+	N.D.	N.D.
		5632C03	N.D.	+	N.D.	N.D.
		5632C04	N.D.	+	N.D.	N.D.
		5632C05	N.D.	+	N.D.	N.D.
2013	5633	5633C01	N.D.	−	N.D.	N.D.
		5633C02	N.D.	−	N.D.	N.D.

N.D. = Not done; + = Positive; − = Negative

94°C, 45 seconds at 60°C, and 2 minutes at 72°C, and were terminated at 72°C for 7 minutes. Then, each DNA fragment was ligated to the pGEM-T easy vector (Promega) and transformed to competent *Escherichia coli* TOP10 (Invitrogen) using the calcium chloride method. Transformants were selected on an LB agar plate containing ampicillin and X-Gal/IPGT. Plasmid was extracted by the QIAprep spin miniprep kit (Qiagen) and sent to SolGent Co. Ltd. (Daejeon, Korea) for DNA sequencing using the dideoxy chain-termination method.

Phylogenetic tree construction: A phylogenetic tree was constructed from nucleotide sequences of the 16S/23S rRNA gene phylogram using the Maximum Likelihood method based on the Kimura 2-parameter model with a bootstrap value based on 1,000 replicates. An additional phylogenetic tree of deduced amino acid sequences of the *ompA* gene was produced by the Maximum Likelihood method based on the JTT matrix-based model. Evolutionary analyses were conducted with MEGA6 version 6.0 software[15].

Table 3. Summary of Chlamydiaceae DNA detected in Siamese crocodiles

Year	Farm ID	Number of tested crocodile	Number of positive crocodile
2012	5504	1	1
2012	5508	2	0
2012	5509[a]	5	5
2012	5510	1	1
2012	5511	2	1
2012	5518	1	1
2012	5520	1	1
2012	5522	2	1
2013	5602	1	1
2013	5606	2	1
2013	5607	1	1
2013	5609[a]	4	4
2013	5620	2	1
2013	5632	4	4
2013	5633	2	0
Total	**14**	**31**	**23 (74.2%)**

[a]farm ID 5509 and 5609 are the same farm but difference year of the sample collection.

Results

Tissue samples of 31 Siamese crocodiles from 14 farms were examined for the presence of Chlamydiaceae by nested PCR. Out of 31 samples, 23 (74.2%) were positive (table 2 and 3). The samples were also tested for mycoplasmas, iridoviruses, and pan-herpesviruses genome detection. For all the samples, genome detection revealed no mycoplasmas, iridoviruses, or pan-herpesviruses, with the exception of sample 5632C03, which was positive for a mycoplasma species (data not shown). The main clinical symptoms of infected crocodiles were depression and anorexia with or without conjunctivitis, diarrhea, and kyphoscoliosis (table 4). Next, the *ompA*, 16S rRNA and 16S/23S rRNA genes of the positive samples were characterized by DNA sequencing. This analysis showed that 21, 13, and 11 of the positive samples were successfully sequenced for the *ompA*, 16S rRNA, and 16S/23S rRNA genes, respectively (table 4). A 1,500-bp fragment of the 16S rRNA gene of the bacteria detected in all crocodiles was similar to that of the family Chlamydiaceae with 95–99% identity. Indeed, the 16S rRNA gene of the bacteria detected in the crocodiles exhibited 98.2–99.3% nucleotide identity with the genera *Chlamydohila* and 95.1–97.8% with the genera *Chlamydia*. Similar to the 16S rRNA gene, the 16S/23S rRNA gene of the bacteria detected in the crocodiles was closer to *Chlamydophila* than *Chlamydia* (97.1–97.5% and 94.8–95.1% nucleotide identity, respectively). The bacterium detected in the same crocodile but from different tissue was also randomly sequenced and compared. The results showed that there was only a 0.05–0.1% difference in the nucleotide sequence with no modification of the amino acid sequence.

A phylogenetic tree of the 16S/23S rRNA phylogram showed that the Siamese crocodile cluster can be grouped together with an 83% bootstrap value and clearly separated from *Cp. caviae* with 100% bootstrap support (Fig. 1). The

Table 4. Detection of Chlamydiaceae DNA using nested PCR and sequencing of three distinct genes from positive samples

Year	Farm ID	Sample ID	Clinical sign	Diagnostic nested PCR	Sequencing PCR		
					ompA	16S rRNA	16S/23S rRNA
2012	5504	5504C01	depress	+	+	−	+
2012	5509	5509C05	anorexia, conjunctivitis, kyphoscoliosis	+	+	−	−
		5509C06	anorexia, conjunctivitis, kyphoscoliosis	+	+	−	−
		5509C07	anorexia, conjunctivitis, kyphoscoliosis	+	+	−	−
		5509C08	anorexia, conjunctivitis, kyphoscoliosis	+	−	−	−
		5509C09	anorexia, conjunctivitis, kyphoscoliosis	+	+	−	−
2012	5510	5510C01	depress, anorexia, jaundice	+	+	+	+
2012	5511	5511C02	anorexia, dry skin, oral ulcer	+	+	−	−
2012	5518	5518C01	depress, anorexia, diarrhea	+	+	+	+
2012	5520	5520C01	depress, anorexia, diarrhea	+	+	+	+
2012	5522	5522C01	anorexia, diarrhea	+	−	−	−
2013	5602	5602C01	no clinical sign	+	+	+	+
2013	5606	5606C01	anorexia	+	+	−	−
2013	5607	5607C02	dwarfism, anorexia	+	+	+	+
2013	5609	5609C01/0	depress, anorexia	+	+	+	−
		5609C01/N	depress, anorexia	+	+	+	−
		5609C02/N	depress, anorexia	+	+	+	−
		5609C03/N	depress, anorexia	+	+	+	+
2013	5620	5620C02	depress, edema	+	+	+	−
2013	5632	5632C02	anorexia	+	+	+	+
		5632C03	anorexia	+	+	+	+
		5632C04	anorexia	+	+	+	+
		5632C05	anorexia	+	+	−	+
		Total		23	21	13	11

+ = Positive; − = Negative

same clustering pattern was reproduced by phylogeny and constructed from deduced amino acid sequences of the *ompA* gene, which encode the major outer membrane protein. However, sequence variation within the bacteria detected in crocodile was expectedly greater and the resulting phylogram was more branched (Fig. 2a). The *ompA* phylogeny revealed the bacteria detected in the Siamese crocodiles are branched into three different clades and that bootstrap values of 96%, 100%, and 98% support the creation of genotypes I, II, and III, respectively (Fig. 2b).

Discussion

Chlamydiaceae is found globally and causes chlamydiosis in humans and animals. The disease was previously reported in saltwater crocodiles in South Africa and Papua New Guinea but there has been no documentation of the disease in freshwater crocodiles, including Siamese crocodiles[10,11]. Approximately 74% of clinical Siamese crocodile samples were found to be positive for the Chlamydiaceae species. Most of the infected crocodiles showed depression and anorexia with or without conjunctivitis, kyphoscoliosis and diarrhea. Although mycoplasmas and herpesviruses have been previously reported as causes of death in crocodiles on farms in

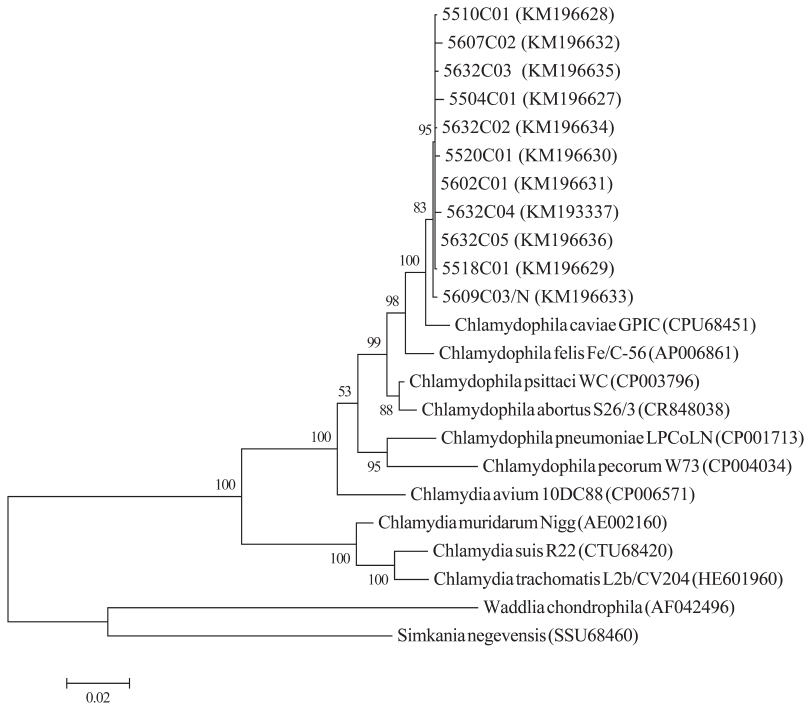
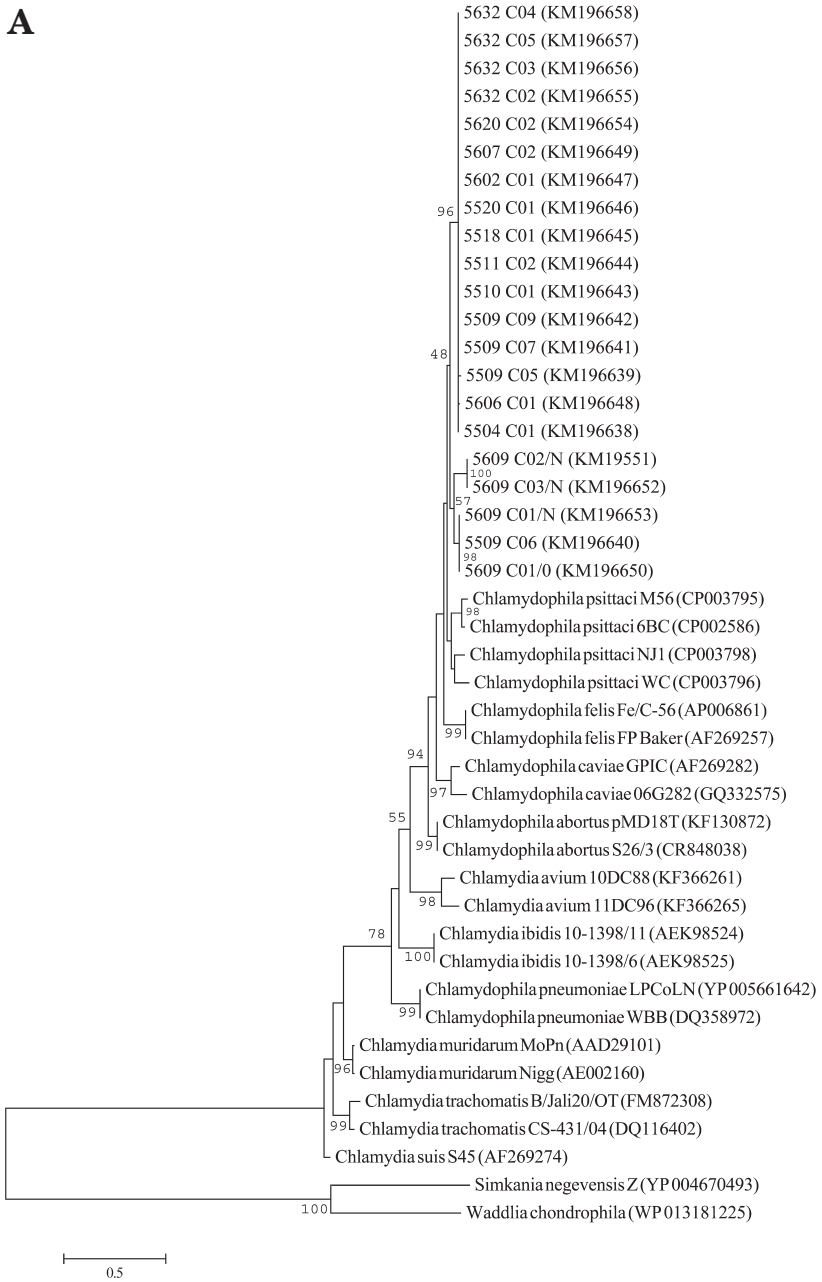


Fig. 1. The 16S/23S rRNA phylogram. A portion of the ribosomal operon called 16S/23S rRNA gene sequence was used for phylogeny construction. Numbers show the percentage of times each branch was found in 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Accession numbers of the sequences are shown in parenthesis.

Zimbabwe[12], they were not found in this study. The present data indicates that infection in the Siamese crocodiles was likely caused by bacteria in the family Chlamydiaceae. The presence of an immunologic condition in the host and the infectious dose of the pathogen can also play a role in the severity of chlamydiosis.

There are two criteria for assignment of the bacterium to the family Chlamydiaceae and the corresponding genus. The first criterion is that strains classified in the family should have more than 90% nucleotide identity with the 16S rRNA gene[6,7]. The second criterion is that strains classified in the same genus should have more than 95% nucleotide identity with the 16S and/or 23S rRNA genes[7]. DNA sequencing and analysis of the bacterium detected in Siamese crocodiles showed that nucleotide identity with 16S rRNA is more than 95%. The 16S rRNA and 16S/23S rRNA genes were found to be closely related to the genus *Chlamydophlila* with more than 97% nucleotide identity. Therefore, these criteria justified the assignment of bacterium in the Siamese crocodile samples to the genus *Chlamydophlila* and the family Chlamydiaceae. The species of the bacterium was characterized by genetic evolution using 16S/23S rRNA and the *ompA* gene loci. A phylogram constructed from a nearly full length of 16S/23S rRNA showed the Siamese crocodile bacterium cluster was grouped together with good statistical support (83%) and diverged far from the *Cp. caviae* cluster with 100% bootstrap support. Use of 16S/23S rRNA has been previously described as a rapid and reproducible method for taxonomical identification and classification of Chamydiaceae strains[6]. In addition, *ompA* phylogeny has also demonstrated the same clustering pattern as 16S/23S rRNA phylogeny, but sequences within the *ompA* gene have more variance, resulting in more clusters in the bacteria detected in the Siamese crocodiles. The *ompA* gene encodes the outer membrane protein (a surface antigen) and responses to immune pressure and allows for antigenic



diversity while maintaining a basic structural function. Thus, it appears to be a candidate for evolutionary and taxonomic analysis[8,2]. Genotyping by *ompA* gene analysis is largely identical to serotyping by the serovar-specific monoclonal antibody test[17]. In the present study, the *ompA*-based phylogram showed that the bacteria detected in Siamese crocodiles were grouped into three different clusters. The branching of these clusters, named genotypes I, II, and III, was strongly supported with 96%, 100%, and 98% bootstrap value, respectively.

In conclusion, this study is the first report of chlamydiosis in Siamese crocodiles and reveals the identification of a suspected new species of the family Chlamydiaceae in Siamese crocodile hosts based on analysis of 16S/23S rRNA and *ompA* gene loci.

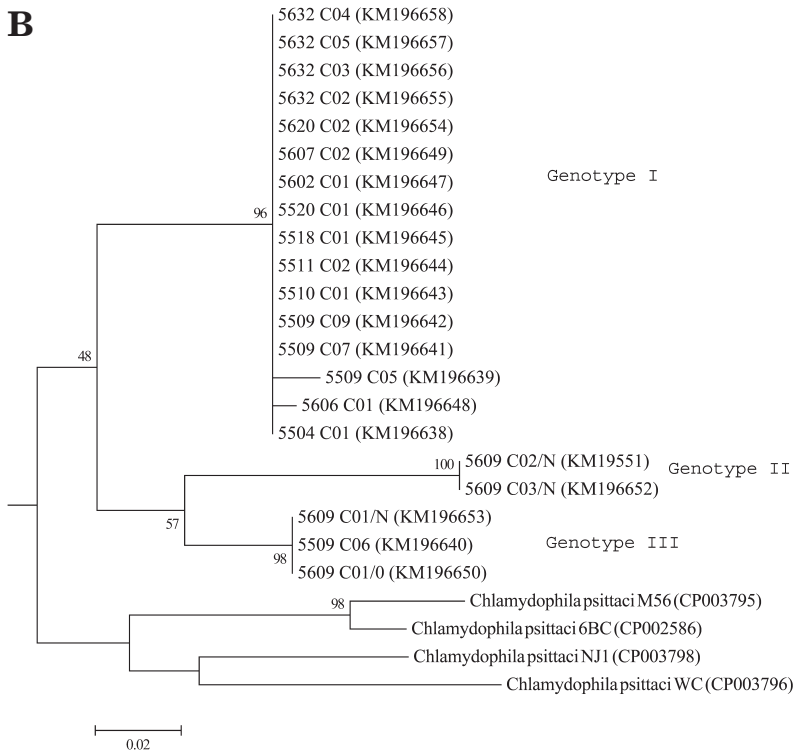


Fig. 2. The *ompA* phylogeny. A: The phylogenetic tree was constructed from putative amino acid sequences of the *ompA* gene from GenBank and Siamese crocodile isolates. B: Detail of a part of a Siamese crocodile branch. Numbers show the percentage of times each branch was found in 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The branches with bootstrap values lower than 45% are not shown.

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