NOTE
Wildlife Science

Molecular prevalence and genotyping of *Chlamydia* spp. in wild birds from South Korea

Jipseol JEONG[1)#], Injung AN[1)#], Jae-Ku OEM[1)], Seung-Jun WANG[1)],
Yongkwan KIM[1)], Jeong-Hwa SHIN[1)], Chanjin WOO[1)], Youngsik KIM[1)],
Seong-Deok JO[1)], Kidong SON[1)], Saemi LEE[1)] and Weonhwa JHEONG[1)*]

[1)]Environmental Health Research Department, National Institute of Environmental Research, Hwangyeong-ro42, Seo-gu, Incheon, Republic of Korea

ABSTRACT. Wild birds are reservoirs for *Chlamydia* spp. Of the total 225 samples from wild birds during January to September 2016 in Korea, 4 (1.8%) and 2 (0.9%) showed positive for *Chlamydia psittaci* and *Chlamydia gallinacea*, respectively. Phylogenetic analyses and comparisons of sequence identities for outer-membrane protein A (*omp*A) revealed that Korean *C. psittaci* fall into three previously known genotypes; genotype E, 1V and 6N, whereas the Korean *C. gallinacea* were classified as new variants of *C. gallinacea.* Our study demonstrates that wild birds in South Korea carry at least two *Chlamydia* species: *C. psittaci* and *C. gallinacea*, and provides new information on the epidemiology of avian chlamydiosis in wild birds.

KEY WORDS: *Chlamydia* spp., genetic diversity, South Korea, wild bird

Chlamydia spp. are the etiological agents of chlamydiosis in animals and humans [16, 22]. The family *Chlamydiaceae* encompasses the single genus *Chlamydia,* which comprises 11 species: *Chlamydia psittaci, C. muridarum, C. suis, C. trachomatis, C. abortus, C. caviae, C. felis, C. pecorum, C. pneumoniae, C. avium* and *C. gallinacea* [22, 24, 26, 27].

Of these species, *C. psittaci* is the most well-known zoonotic agent [13]. Isolates of *C. psittaci* have been reported to exist in approximately 460 species of birds [15]. *C. psittaci* infection in birds can persist for months to years, often without causing obvious illness [16]. Until recently, there were nine outer-membrane protein A (*omp*A) genotypes described in *C. psittaci*, designated A–F, E/B, M56 and WC [25], as well a number of provisional genotypes (1V, YP84, R54, 6N, CPX0308, I and J), representing strains that have thus far been un-typeable [20, 25]. The importance of genotyping lies in the fact that certain genotypes tend to be associated with certain hosts and differ in virulence [13]. Genotypes A and B are usually associated with psittacine birds and pigeons, respectively. Genotype C is primarily isolated from ducks and geese, and genotype D from turkeys. Genotype E is the most divergent among these types, which has been isolated from pigeons, ratites, ducks and turkeys, and occasionally from humans [13]. Regarding to *C. psittaci* in South Korea, association with ocular adnexal mucosa-associated lymphoid tissues (MALT) lymphoma in human has been reported [31]. Interestingly, this association between *C. psittaci* and the MALT lymphoma seems to show geographic variation. For example, *C. psittaci* was detected in the MALT patients in Italy and South Korea, but not in the patients in the Northern China and Japan [3].

To date, *C. gallinacea* has been mainly isolated from chickens, ducks, guinea fowl, turkeys and other domestic poultry in China and European countries [12, 32]. This new emerging agent was predominantly found in asymptomatic poultry; however, a decrease in the rate of weight gain was reported in *C. gallinacea*-infected chickens [12]. So far, at least 13 genotypes of *omp*A protein of *C. gallinacea* have been identified. Because it has been suggested that *C. gallinacea* may cause a potential zoonotic infection [18], the pathogenicity and characterization of each genotype require systemic investigation [12].

Although studies involving the genetic characterization of *Chlamydia spp.* are prevalent on a global scale [8, 17, 23, 30], there is little information on the occurrence or epidemiology of *Chlamydia* spp. infection in wild birds of South Korea. In the present study, polymerase chain reaction (PCR) was performed to obtain baseline data on the prevalence of *Chlamydia* spp. in various species of wild birds. We also performed sequence analysis of 16S rRNA and *omp*A genes to evaluate the genetic diversity and epidemiology of *Chlamydia* spp. in wild birds in South Korea.

*Correspondence to: Jheong, W., Environmental Health Research Department, National Institute of Environmental Research, Hwangyeong-ro 42, Seo-gu, Incheon, 22689, Republic of Korea. e-mail: czar21@korea.kr
#Theses authors contributed equally to this work.

Bird carcasses were obtained from wildlife rescue and conservation centers in Busan, Chungbuk, Daejeon, Gangwon, Gyeongnam, Jeonbuk, Jeonnam and Ulsan during January to September 2016. Tracheal swabs of bird carcass were collected individually from 225 birds, from 43 species belonging to 14 orders (Supplemental Table 1). Necropsies were performed on all dead birds. Tissue samples including lungs, spleens and livers were aseptically collected and examined for macroscopic lesions, including multifocal hepatic necrosis and spleen and liver enlargement. All swabs were immediately placed in BD Universal Viral Transport (UVT) tubes (BD Biosciences, Baltimore, MD, U.S.A.). Swabs were stored at −80°C until DNA extractions were performed.

DNA was extracted from tracheal swabs, using the automated Maxwell RSC Viral Total Nucleic Acid Purification Kit with the Maxwell 16 instrument (Promega Corporation, Madison, WI, U.S.A.). All steps were automated according to the pre-programmed viral nucleic acid protocol. An initial screening was performed with whole genomic DNAs by 23S rRNA-based, *Chlamydia* spp. -specific quantitative real time PCR with QuantStudio 6 flex (Thermo Scientific, Rockford, IL, U.S.A.), as described in a previous report [5, 23]. For further characterization of chlamydial *omp*A and 16S rRNA, fragments were amplified using previously described representative primer pairs and conditions: CTU/CTL (*omp*A sequencing) [4] and 16S1/rp2 (16S rRNA sequencing) [21]. PCR-amplified segments of *omp*A and 16S rRNA genes were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and directly sequenced (Macrogen, Seoul, Korea). Nucleotide sequences have been deposited under GenBank nos. KX603684-KX603697.

Nucleotide sequences were BLASTed against the NCBI database to identify related sequences and aligned using CLUSTALW2 [6]. The multiple alignments were used to infer the phylogenies with the maximum-likelihood (ML) method implemented in MEGA 5 [28]. To obtain the ML tree topologies, 1,000 bootstrap replicates were performed for each dataset. The inferred tree topologies were inspected visually using FigTree version 1.3.1.

Chlamydia prevalence was defined as the number of PCR-positive samples described as a percentage of the total sample number. No macroscopic lesions were detected during the necropsy of 225 birds from wildlife rescue and conservation centers. However, six of 225 tracheal swabs tested positive for chlamydial DNA by a diagnostic real-time PCR assay for 23S rRNA, corresponding to 2.7% (6/225) of the examined population. Birds that tested positive for *Chlamydia* spp. belonged to four species in three orders (Charadriiformes, Columbiformes and Passeriformes), including woodcock (*Scolopax rusticola*), domestic pigeon (*Columba livia var. domestica*), the Korean magpie (*Pica pica sericea*) and rook (*Corvus frugilegus*) (Supplemental Table 1). Geographically, the *Chlamydia* spp. were detected in Jeonbuk province (one case in domestic pigeon) and Ulsan metropolitan city (two cases in woodcock, one case in Korean magpie and two cases in rook) (Fig. 1).

The confirmatory PCR targeting the variable domains of 16S rRNA genes enabled us to identify the presence of two *Chlamydia spp*. in the birds examined: *C. psittaci* (1.8%; 4/225) and *C. gallinacea* (0.9%; 2/225) (Table 1).

The *Chlamydia* 16S rRNA sequences from nier-A97, -A101 and -A113 were identical and displayed a high degree of conservation with that of *C. psittaci* strain Prk46 (AB001809, 99.8% identity), which represents an intermediate variant between *C. psittaci* and *C. abortus.* The sequence of nier-A124 is 100% identical to that of previously reported human and pigeon *C. psittaci* isolates. The nucleotide sequences of *C. gallinacea* (nier-A94 and -A186) shared 99.9% identity and were closely related to those of *C. gallinacea* 08-1274/13 (GQ398027, 99.0% identity). A comparative phylogenetic analysis based on the 16S rRNA sequence demonstrated that the *C. psittaci* and *C. gallinacea* strains detected in this study are all closely related to their representative *C. psittaci* and *C. gallinacea* (Fig. 2A).

The *omp*A sequences of *C. psittaci* and *C. gallinacea* were amplified by PCR to analyze genetic diversity. The *omp*A sequences of nier-A97 and -A101 displayed a high level of nucleotide identity (99.2%) and conservation with *omp*A nucleotide sequences from genotype 1V (EF028916) isolates from Russian hooded crows (98.5% and 99.3% identity, respectively). In addition, nier-A113 showed a high degree of identity (98.9%) to genotype 6N (EF197820) isolates from Russian rooks [30]. The nier-A124 nucleotide sequence was 100% identical to that of genotype E isolate MN (AF269262), which has been isolated from humans and a variety of birds worldwide [1, 11]. A comparative phylogenetic analysis based on the *omp*A sequence revealed that the four *C. psittaci* sequences identified in this study are all closely related to their respective *C. psittaci* genotypes (Fig. 2B).

The two *C. gallinacea* sequences (nier-A94 and -A186) were 100% identical and showed 85.5% nucleotide identity to the sequence of *C. gallinacea* isolate 11-1879 from Croatia, which was the most closely related to the sequence of the previously characterized *C. gallinacea* isolates. A phylogenetic comparison showed that the *omp*A genes identified from two instances of *C. gallinacea* in this study clearly diverged to the previously reported *C. gallinacea* isolates from France and China (Fig. 2C).

To the best of our knowledge, our study is the first to characterize avian chlamydiosis in wild birds from South Korea. Diagnostic real-time PCR-based approaches for the detection of *Chlamydia* spp. from tracheal swab samples have been previously employed for 23S rRNA [5, 23]. In the present study, two *Chlamydia* species [*C. psittaci* (in 4/225) and *C. gallinacea* (in 2/225)] were detected, and their genetic variants were identified by analyzing the sequences of *omp*A and 16S rRNA.

In the present study, *C. psittaci* was detected in domestic pigeons, but did not appear to be associated with any signs of disease in these birds on necropsy. Thus, these birds could be asymptomatic carriers of *C. psittaci*. Studies describing the *C. psittaci* carrier status of the pigeon population have been published worldwide, thereby suggesting their potential zoonotic aspects [14, 29]. For example, the prevalence of *C. psittaci* was 7.9% (26/331) and 22.2% (103/463) in feral pigeons in Netherland and Japan, respectively. Sequence analyses of *omp*A and 16S rRNA revealed that *C. psittaci* is identical to genotype E, which was previously detected in both humans and pigeons worldwide [1]. Although our results showed a relatively low prevalence of *C. psittaci* in pigeons compared to that observed in other countries, this finding indicates that the pigeon could also be a natural vector for *C. psittaci* genotype E in South Korea and a zoonotic threat in the region.

C. psittaci genotypes 1V and 6N were first discovered in corvid species (crow and rook) in Russia in 2006 [30]. The discovery

Table 1. *Chlamydia* spp. identification in South Korea from January to September 2016

Sample ID	Species	Region	*Chlamydia* spp. identification by 16s rRNA	Genotyping by *omp*A gene
nier-A94	Woodcock	US	*C. gallinacea*	Un-typeable
nier-A97	Rook	US	*C. psittaci*	Genotype 1V
nier-A101	Korean Magpie	US	*C. psittaci*	Genotype 1V
nier-A113	Rook	US	*C. psittaci*	Genotype 6N
nier-A124	Domestic pigeon	JB	*C. psittaci*	Genotype E
nier-A186	Woodcock	US	*C. gallinacea*	Un-typeable
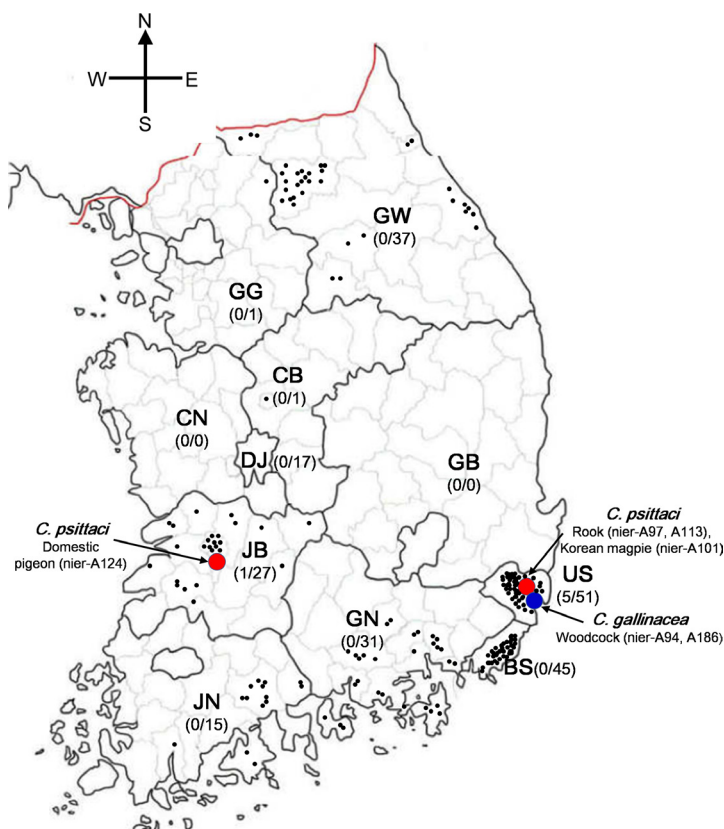
Fig. 1. *Chlamydia* spp. prevalence in wild birds in South Korea. The red and blue filled circles with arrows indicate *C. psittaci* and *C. gallinacea*, respectively. The black filled circles indicate *Chlamydia* negative samples. GG, Gyeonggi province; GW, Gangwon province; CN, Chungnam province; DJ, Daejeon metropolitan city; CB, Chungbuk province; JB, Jeonbuk province; JN, Jeonnam province; GB, Gyeongbuk province; GN, Gyeongnam province; US, Ulsan metropolitan city; BS, Busan metropolitan city.

of *C. psittaci* genotypes 1V and 6N in the rooks and Korean magpies in the present study provides evidence of circulation of *C. psittaci* in the corvid species of our region, where the rook may be an asymptomatic carrier. The rook is characterized as a migratory bird, which breeds in central Europe or Siberia and migrates to South Korea or Japan during the winter season [2]. In particular, Taehwa River located in Ulsan metropolitan city is one of the most important resting sites for migratory rooks, which overwinter in South Korea from Siberia. The *omp*A sequences of *C. psittaci* genotypes 1V and 6N from rooks in our study showed a close association with those of isolates from Russian corvid species, although the 16S rRNA sequence of our isolates showed a close genetic similarity to *C. psittaci* prk/46, which is known to cause systemic infections in parakeets in Japan [9] (Fig. 2A and 2B). These results indicate that the migratory rook between Russia and South Korea plays an important role in the epidemiology of *C. psittaci* genotypes 1V and 6N.

This study presents the first instance of detection of *C. gallinacea* in South Korea. *C. gallinacea* was reported in chickens during an outbreak of psittacosis in Germany [10] and was subsequently isolated from chickens in France (*C. gallinacea* strain 08-1743/3) and Croatia (*C. gallinacea* strain 11-1879). In France, a slaughterhouse worker presenting with atypical pneumonia was reportedly exposed to *C. gallinacea*-carrying chickens [18]. The *C. gallinacea* in our study appeared to be segregated genetic variants in comparison to known isolates from China and Europe, based on a phylogenetic analysis of the *omp*A sequences (Fig.

A
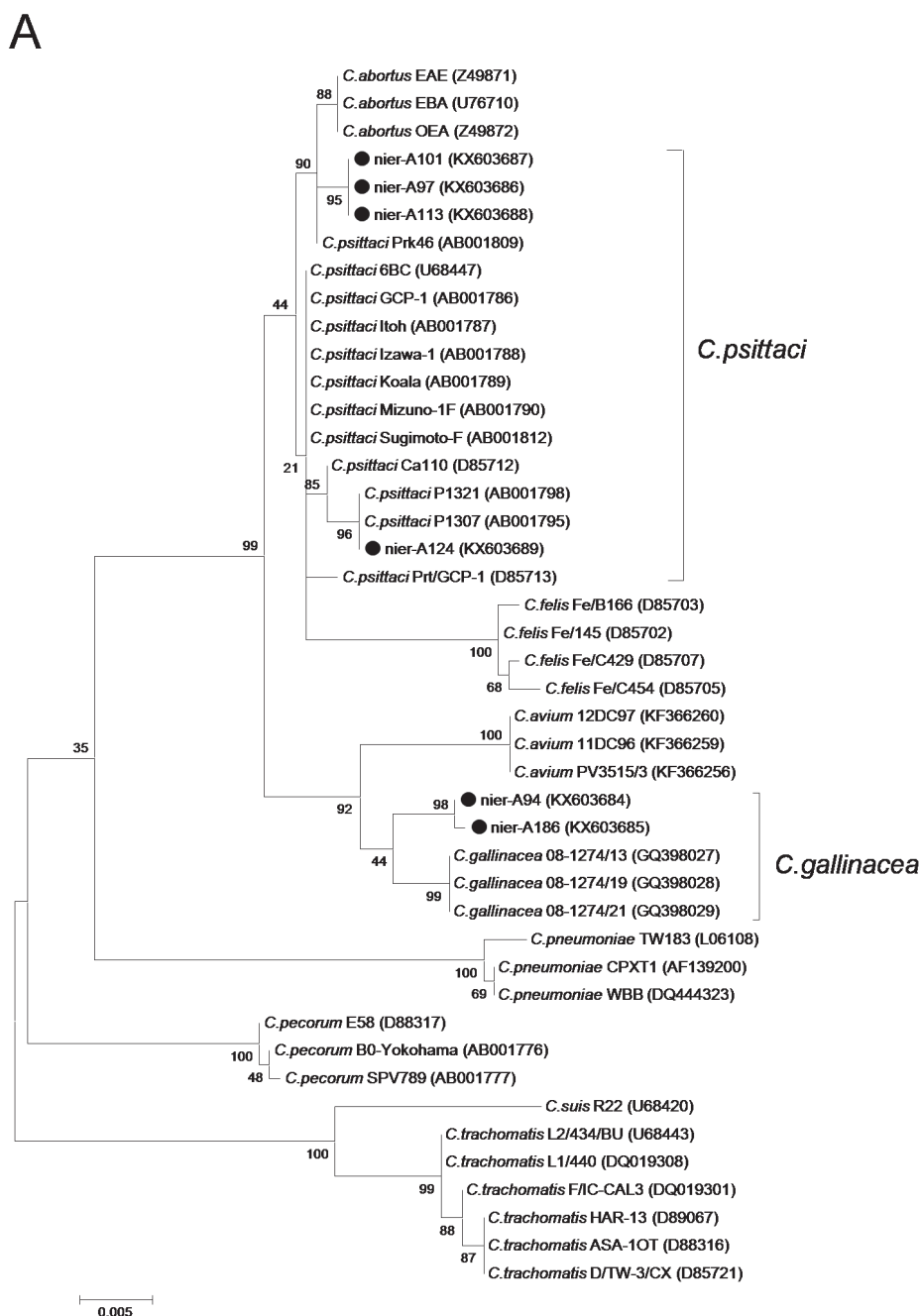
Fig. 2. Phylogeny of *Chlamydia* spp. A. The 16S rRNA gene (1,370 bp) of *Chlamydia* spp. B. The *omp*A gene (>886 bp) of *C. psittaci*. C. The *omp*A gene (>379 bp) of *C. gallinacea*. The phylogenetic distances were calculated using MEGA 5 with the maximum-likelihood algorithm, and the trees were visualized using FigTree 1.3.1. Bootstrap values are shown as percentages of 1,000 replicates. Bootstrap value less than 50% is shown in the phylogenetic trees. The scale bars represent the number of substitutions per site. Nucleotide sequences in the present study are indicated by black dots.

2C). Our findings are in agreement with those in previous reports, which showed that geographically separated isolates displayed genetic diversity (85.7% similarity between isolates in China and Europe) [12, 18]. Furthermore, the partial sequencing of the 16S rRNA and *omp*A gene revealed that although the 16S rRNA sequences in our study were similar to those of previous *C. gallinacea* isolates (99% nucleotide identity), the *omp*A genes were highly distinguished (85.5% nucleotide identity). This is in agreement with a previous report, in which rRNA genes were subjected to evolutionary pressure to a far lesser extent than genes encoding outer membrane proteins [7].

Although the prevalence of *C. gallinacea* in woodcocks was very high (2/3, 66.7%), the sample size was too low for statistical analysis. Because our method of sample collection from wildlife health centers could have contributed to a bias, additional studies are required to assess the prevalence of *C. gallinacea* in woodcocks. Furthermore, because it was reported that bovine *C. gallinacea*
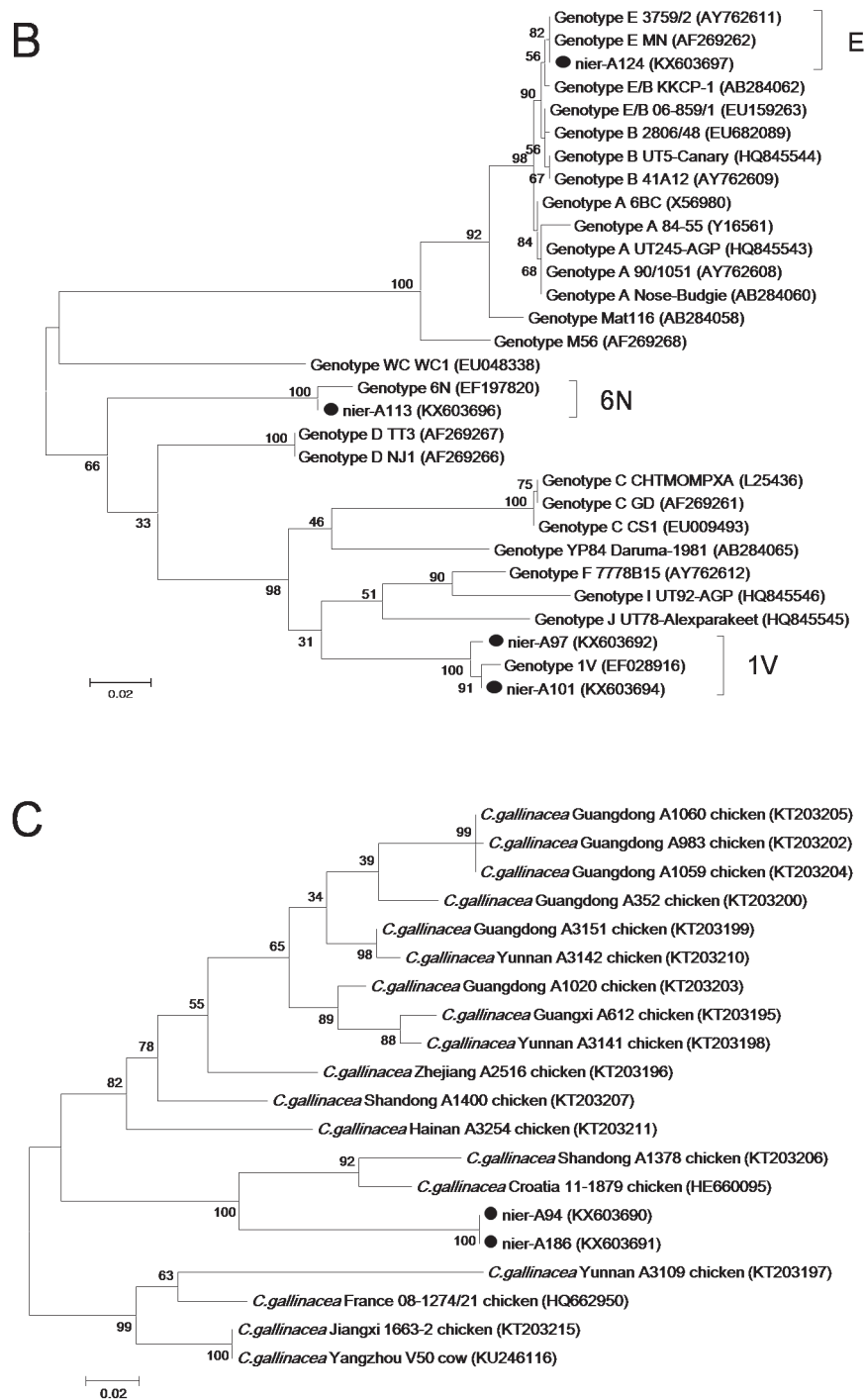
B

C

Fig. 2. Continue

had *omp*A sequences identical to those of avian species in China [19], additional studies should be conducted to understand the potential transmission of *C. gallinacea* between wild birds and domestic animals, including poultry and mammals, in Korea.

In conclusion, this study demonstrates the prevalence of *Chlamydia* spp. in wild birds, as well as the genetic diversity of *C. psittaci* and *C. gallinacea,* in South Korea. *Chlamydia* spp. in wild birds could be a potential source of infection in domestic animals and humans in South Korea, especially in people associated with handling of wild birds; hence, it is necessary to implement biosecurity measures to minimize the possibility of infection. The results obtained from this study contribute to improving the present understanding of the epidemiology of avian chlamydiosis in wild bird populations.

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