



HAL
open science

**Evidence for the existence of a new genus
Chlamydiifrater gen. nov. inside the family
Chlamydiaceae with two new species isolated from
flamingo (*Phoenicopterus roseus*): *Chlamydiifrater
phoenicopteri* sp. nov. and *Chlamydiifrater volucris* sp.
nov.**

F. Vorimore, M. Hölzer, E.M. Liebler-Tenorio, L.-M. Barf, S. Delannoy, M. Vittecoq, R. Wedlarski, A. Lécu, S. Scharf, Yannick Blanchard, et al.

► **To cite this version:**

F. Vorimore, M. Hölzer, E.M. Liebler-Tenorio, L.-M. Barf, S. Delannoy, et al.. Evidence for the existence of a new genus *Chlamydiifrater* gen. nov. inside the family Chlamydiaceae with two new species isolated from flamingo (*Phoenicopterus roseus*): *Chlamydiifrater phoenicopteri* sp. nov. and *Chlamydiifrater volucris* sp. nov.. Systematic and Applied Microbiology, 2021, 44 (4), pp.126200. 10.1016/j.syapm.2021.126200 . anses-04424879

HAL Id: anses-04424879

<https://anses.hal.science/anses-04424879>

Submitted on 22 Jul 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Evidence for the existence of a new genus *Chlamydiifrater* gen. nov.**
2 **inside the family *Chlamydiaceae* with two new species isolated from**
3 **flamingo (*Phoenicopterus roseus*): *Chlamydiifrater phoenicopteri* sp.**
4 **nov. and *Chlamydiifrater volucris* sp. nov.**

5 Vorimore F¹*, Hölzer M², Liebler-Tenorio EM³, Barf L-M⁴, Delannoy S⁵, Vittecoq M⁶, Wedlarski R⁷,
6 Lécu A⁸, Scharf S³, Blanchard Y⁹, Fach P⁵, Hsia RC¹⁰, Bavoil PM¹¹, Rosselló-Móra R¹², Laroucau
7 K¹, Sachse K⁴.

8 ¹ University Paris-Est, Anses, Animal Health Laboratory, Bacterial Zoonoses Unit, Maisons-Alfort, France.

9 ² Robert Koch Institute, MF1 Bioinformatics, Berlin, Germany.

10 ³ Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Institute of Molecular Pathogenesis, Germany.

11 ⁴ Friedrich-Schiller-Universität Jena, RNA Bioinformatics and High-Throughput Analysis, Jena, Germany.

12 ⁵ University Paris-Est, Anses, Food Research Laboratory, IdentityPath platform, Maisons-Alfort, France.

13 ⁶ Tour du Valat, Centre de recherche pour la conservation des zones humides méditerranéennes, Le Sambuc, Arles, France.

14 ⁷ Bioparc - Zoo de Doué la fontaine, 103 rue de Cholet, 49700 Doué la Fontaine, France

15 ⁸ Parc Zoologique de Paris, avenue de Daumesnil, 75012 Paris, France.

16 ⁹ Unit of Viral Genetics and Biosafety, ANSES, Laboratory of Ploufragan, Ploufragan, France.

17 ¹⁰ University of Maryland, Electron Microscopy Core Imaging Facility, Baltimore, MD, 21201 USA.

18 ¹¹ University of Maryland, Department of Microbial Pathogenesis, Baltimore, MD, 21201 USA.

19 ¹² Marine Microbiology Group, Department of Animal and Microbial Biodiversity, Mediterranean Institute for Advanced Studies, 07190, Esporles,
20 Spain.

21

22

23 *Corresponding author. Phone: (33) 1 49 77 13 00, Fax: (33) 1 49 77 13 44, E-mail address: fabien.vorimore@anses.fr

24

25

26 **Abstract**

27 The family *Chlamydiaceae* currently comprises a single genus *Chlamydia*, with 11 validly published species and seven
28 more taxa. It includes the human pathogens *Chlamydia (C.) trachomatis*, *C. pneumoniae* and *C. psittaci*, a zoonotic
29 agent causing avian chlamydiosis and human psittacosis, as well as other proven or potential pathogens in ruminants,
30 birds, snakes, reptiles and turtles. During routine testing of 15 apparently healthy captive flamingos in a zoo in 2011, an
31 atypical strain of *Chlamydiaceae* was detected by real-time PCR of cloacal swab samples. Sequence analysis of the
32 16S rRNA gene revealed high similarity to the uncultured *Chlamydiales* bacterium clone 122, which previously had
33 been found in gulls. As more samples were collected during annual campaigns of the flamingo ringing program in
34 southern France from 2012 to 2015, *Chlamydiaceae*-specific DNA was detected by PCR in 30.9% of wild birds. From
35 these samples, three strains were successfully grown in cell culture. Ultrastructural analysis, comparison of 16S and
36 23S rRNA gene sequences, whole-genome analysis based on *de novo* hybrid-assembled sequences of the new
37 strains as well as subsequent calculation of taxonomic parameters revealed that the relatedness of the flamingo

38 isolates to established members of the family *Chlamydiaceae* was sufficiently distant to indicate that the three strains
39 belong to two distinct species within a new genus. Based on these data, we propose the introduction of *Chlamydiifrater*
40 gen. nov., as a new genus, and *Chlamydiifrater phoenicopteri* sp. nov. and *Chlamydiifrater volucris* sp. nov., as two
41 new species of the genus.

42

43 Keywords : *Chlamydiifrater phoenicopteri*; *Chlamydiifrater volucris*; *Chlamydiaceae*; Hybrid de novo assembly; 16S
44 rRNA gene sequence; Comparative genome analysis

45

46 Introduction

47

48 The family *Chlamydiaceae* currently comprises a single genus, *Chlamydia*, with 11 validly published species and an
49 additional seven proposed species, four of which at *Candidatus* rank. These obligate intracellular bacteria include the
50 zoonotic agent *Chlamydia (C.) psittaci*, as well as the recently discovered *C. avium*, *C. gallinacea* and *C. buteonis* that
51 all have a preference for avian hosts [42]. These four avian chlamydiae are now easily identifiable thanks to molecular
52 tools designed for their rapid and specific detection [35, 54, 55]. However, several studies have reported the detection
53 of non-classified chlamydial organisms in avian samples. Since chlamydiae have been detected in 465 different bird
54 species [18], it is not surprising that our knowledge on the diversity of chlamydial species occurring in wild birds is
55 steadily expanding. For instance, isolation of two related strains from ibis specimens led to the proposal of *Candidatus*
56 *C. ibidis* as an additional genus member [51]. A new species found in hawks, *C. buteonis*, has just been reported [23].
57 In addition, *C. abortus* strains were recently isolated from wildfowl [48]. From non-avian hosts, recent descriptions of
58 new taxa include *C. serpentis* and *C. poikilothermis* from snakes [46], the *Candidatus* species of *C. sanzinia* and *C.*
59 *corallus* from reptiles [49], as well as *C. testudinis* from tortoises [22].

60 The high genetic diversity of chlamydial strains of avian origin belonging to the order *Chlamydiales* has been
61 mentioned in various studies [1, 3, 17, 56]. The main obstacle to comprehensive genetic characterization of these
62 chlamydial organisms is their obligate intracellular lifestyle, which requires cell culture for recovery of isolates. As
63 numerous failed attempts in various experienced laboratories have shown, these atypical chlamydial strains are
64 extremely difficult to grow. As a consequence, most of the studies published so far have been unable to classify and
65 comprehensively characterize atypical avian chlamydial organisms.

66 In the present paper, we report the identification and description of new chlamydiae from captive and wild flamingos.
67 Initially, diagnostic testing during their introduction into a zoo revealed the occurrence of an atypical chlamydial agent.
68 Follow-up investigations led to the isolation and detailed characterization of three chlamydial strains, which will be
69 classified as two new species belonging to a new genus of the family *Chlamydiaceae*.

70

71 Materials and Methods

72

73 Samples

74 Cloacal swabs were taken from both captive (*Phoenicopterus chilensis*) and wild birds (*Phoenicopterus roseus*).
75 Captive flamingos sampled in 2011 were from the Bioparc zoo (Doué-la-Fontaine, France) (n=18) and those sampled

76 in 2014 were from the Paris Zoo (n=37). Wild specimens (n= 404) were collected during the annual round-ups that are
77 organized each summer in the Camargue region of France for the purpose of banding flamingos chicks. In 2012, 2013
78 and 2015, catches took place in the Étang du Fangassier, whereas in 2014, due to the nesting change of the group,
79 they took place in the saline of Aigues-Mortes near the Mediterranean coast of Southern France.
80 Duplicate swabs were collected from each bird. The first swab was stored dry at -20°C until being subjected to DNA
81 extraction and the other was stored in SPG medium [44] at – 80°C until inoculation for isolation. Key data are
82 summarized in **Table 1**.

83

84 ***Direct detection of Chlamydiaceae DNA from avian samples***

85 Cloacal swabs were subjected to DNA extraction using a QIAamp DNA Mini Kit, following the buccal swab protocol
86 (Qiagen, Courtaboeuf, France). DNA was eluted with 150 µL of AE buffer and stored at –20°C before analysis. A
87 *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene was used in this study [9]. An exogenous positive
88 control was included in each sample to test for the presence of PCR inhibitors (Exogenous internal positive control,
89 TaqMan, Life technologies). All samples with a quantification cycle (Cq) over 40 were considered negatives.
90 PCR assays specifically identifying *C. psittaci*, *C. avium*, *C. gallinacea* or *Candidatus C. ibidis* species were conducted
91 as previously described [35, 54, 55].

92

93 ***Isolation and propagation in cell culture***

94 Buffalo green monkey (BGM) cells in minimal essential medium (MEM, Lonza, France) with 5% fetal calf serum were
95 seeded into Trac bottles containing glass coverslips (Thermo Scientific, France) and incubated at 37°C with 5% CO₂ in
96 a fully humidified cabinet for 2 days. Swabs in 2 mL of SPG medium were ultrasonicated (ten 0.8s pulses at an
97 amplitude of 80%; Branson 450D Sonifier) and medium (30 to 300 µL) was inoculated into six Trac bottles with
98 confluent grown BGM monolayers. After inoculation, the bottles were centrifuged at 3000 g for 60 min at 37°C and
99 subsequently incubated for 2 h in the CO₂ cabinet. The MEM medium was then replaced with serum-free medium
100 UltraMDCK (Lonza, France) containing the antibiotics amphotericin B (2.5 µg/mL), gentamicin (10 µg/mL) and
101 vancomycin (25 µg/mL). Medium was renewed at 18 h post infection. Three days after inoculation, a single coverslip
102 was fixed with methanol, and the monolayer was stained with IMAGEN Chlamydia (Oxoid, France). A sample was
103 considered positive when inclusions of typical chlamydial morphology appeared as bright apple-green spots after a
104 maximum of two passages.

105

106 ***Transmission electron microscopy (TEM)***

107 BGM cells were infected with isolates 14-2711_R47 and 15-2067_O50. Infected cells were harvested at 24, 48 and 72
108 hours post-infection. After washing, cells were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) with
109 1.8% glucose for 6 h at 4°C. Then they were gently scrapped off the culture plate, transferred into an Eppendorf tube
110 and the fixative replaced by cacodylate buffer. For embedding, cells were centrifuged for 5 min at 1.500 x g. The cell
111 pellet was embedded in 2% agarose and sectioned to 1 mm³ cubes. Cubes were post-fixed in 2% osmium tetroxide,
112 dehydrated in acetone and embedded in araldite Cy212. Relevant areas were selected in Toluidine blue-stained semi-

113 thin sections. Ultrathin sections (85 nm) were stained with uranyl acetate and lead citrate, examined by transmission
114 electron microscopy (Tecnai 12; FEI) and representative micrographs documented (TEMCAM FX416, TVIPS, Gauting,
115 Germany).

116

117 ***DNA-based characterization***

118 Amplifications and 16S rRNA sequence analysis

119 Primers 16S1 and rp2 were used for amplification of approximately 1500 nucleotides of the 16S rRNA gene as
120 previously described [41]. Sequences were combined in an alignment with previously published sequences relevant for
121 phylogenetic and epidemiological studies. The first 16S rRNA sequence obtained from sample 11-261_B096 was
122 searched (BLASTN) against the NCBI non-redundant nucleotide database for identification. Other sequences were
123 analyzed using the Bionumerics software package version 7.6.2 (Applied-Maths, Belgium). A similarity matrix was
124 calculated using a pairwise alignment with a Jukes and Cantor correction. A phylogenetic tree was constructed using
125 RAxML v8 [45], with the GTRGAMMA model and 1000 bootstrap replicates based on an alignment of all 16S rRNA
126 sequences using MAFFT v7 [19]. The 16S rRNA nucleotide sequences of samples 11-261_B96, 13-234_I89, 13-234
127 I94, 13-234_I99, 13-3212_BE60, 14-2711_R45, 14-2711_R47_isolate, 14-2711_R79, 15-2711_O50_isolate, 15-
128 2711_O77, 15-2711_O99_isolate have been deposited under accession numbers LR777664 to LR777674 at the ENA
129 database.

130

131 DNA preparation for genome sequencing

132 Genomic DNA of isolates 14-2711_R47, 15-2067_O50 and 15-2067_O99 was prepared from 14 mL of BGM cell
133 culture harvested from a 75-cm² culture flask. Each suspension was centrifuged at 20,000 g at 8°C for 45 min, and the
134 supernatant was discarded. The pellet was re-suspended in 40 mL of PBS buffer and centrifuged at 500 g for 15 min to
135 remove cell debris. The supernatant was transferred to a fresh tube and high-speed centrifuged as above. The pellet
136 was re-suspended in 200 µL of PBS. Prior to DNA extraction, a DNase I (NEB, France) treatment was performed to
137 remove potential DNA from cells. Genomic DNA was extracted using the Qiamp DNA Mini kit (Qiagen, France), with an
138 additional RNase A (Roche) treatment after lysis, according to the cell protocol of the manufacturer. DNA was eluted in
139 50 µL of water and re-purified using the NEBNext Microbiome DNA enrichment kit (NEB, France).

140

141 Genome sequencing, hybrid *de novo* assembly, annotation and comparative analysis

142 Libraries were prepared using the Nextera XT kit (Illumina) and the Native barcoding genomic DNA kit (Oxford
143 Nanopore Technologies; ONT). Whole-genome sequencing was performed using the Illumina MiSeq platform and the
144 MinION with a R9.4.1 flowcell according to the manufacturers' instructions. The MiSeq run was carried out on the DNA
145 preparation, yielding paired-end reads of 150 bp using MiSeq V2 reagents. Nanopore raw signal Fast5 files were
146 basecalled and then demultiplexed using Guppy v3.4.4 (ONT). A *de novo* hybrid assembly was done with long reads
147 from MinION and short reads from Illumina using Unicycler v0.4.8 with default parameters [52]. Resulting contigs were
148 subsequently blasted on the nucleotide database of NCBI to remove residual mammalian sequences from cell culture
149 constituents. Assembled genomes were annotated using Prokka v1.14.5 [43]. Short reads from Illumina were mapped

150 back to the assembly using BWA v0.7.12-5 [25] and long reads from Nanopore were mapped with minimap2 v2.17-
151 r941 [24]. Statistics of the assemblies were generated using Qualimap v2.2.2-dev [10]. Genome sequences of all three
152 isolates have been deposited in the ENA database under the following accession numbers: ERZ1300893 (14-
153 2711_R47), ERZ1300894 (15-2067_O50), and ERZ1300895 (15-2067_O99). Illumina raw reads are available at ENA
154 under accession numbers ERR3950885 (14-2711_R47), ERR3950886 (15-2067_O50), and ERR3950887 (15-
155 2067_O99) and ONT raw reads under accession numbers ERR3950922 (14-2711_R47), ERR3950921 (15-
156 2067_O50), and ERR3950920 (15-2067_O99).

157 For comparative genome analysis, the novel Roary ILP Bacterial Annotation Pipeline (RIBAP) was used
158 (github.com/hoelzer-lab/ribap). Based on annotations provided by Prokka, the pipeline combines various core gene
159 sets calculated by Roary [34] with a newly adapted Integer Linear Programming (ILP) algorithm [28]. The combination
160 of Roary and ILP results led to the creation of the final interactive RIBAP table, which allows systematic searches for
161 common and specific features in the genomes, as well as downloads of multiple-sequence alignments and
162 phylogenetic trees.

163 RIBAP is implemented using the workflow management system Nextflow [6] and all used tools are encapsulated in
164 Docker images [30] to allow easy installation and execution of the pipeline (see GitHub for further instructions). We ran
165 the pipeline in v0.3 from a set of three genomes of the new flamingo isolates and twelve genomes of selected type and
166 reference strains of *Chlamydia* spp. All 15 input genomes and the interactive RIBAP output table are available at the
167 Open Science Framework (<https://osf.io/d8yk3>). The following command was used to execute the pipeline: `nextflow run`
168 `hoelzer-lab/ribap -r v0.3 --fasta '*.fasta'`. The POCP values and the UpSet plots described below are based on the
169 RIBAP output and the defined homologous groups.

170

171 UpSet diagrams

172 The UpSetR package is a scalable tool for visualizing intersecting sets and their properties [4]. As an input for UpSetR,
173 we selected the homologous gene groups identified with RIBAP for the 15 input genomes. The UpSet visualization of
174 all 15 strains was restricted to the largest 40 intersecting sets.

175

176 Real-time PCR for detection of the new genus and the two new species

177 A new real-time PCR was designed to specifically detect the members of the new genus. Briefly, primers Chlfr_F (5'-
178 ACCTGGTACGCTCAAGTTTC -3'), Chlfr_R (5'- GCGTGTGTGATGAAGGCTTT -3') and probe Chlfr_P (5'-FAM-
179 TCCCTATTCCCAGCGAAA -TAMRA-3') targeting the 16S rRNA gene locus were used in a TaqMan assay. Real-
180 time PCR was performed using the 2x TaqMan Fast Advanced Master Mix supplemented with ROX (Applied
181 Biosystems), 0.625 μ M of each primer, 0.1 μ M of probe and 2 μ l of DNA sample in a 20- μ l final volume.

182 Amplification was carried out on a Viia 7 thermocycler (Applied Biosystems) using the following cycling parameters:
183 initial step 50°C for 2 min followed by heating at 95 °C for 20 s, and 45 cycles of 95 °C/3 s and 61 °C/30 s. The
184 quantification cycle (Cq) was calculated automatically. In addition, two real-time PCRs were designed to detect each
185 species of the new genus. For the new species comprising isolate 14-2711_R47, primers Chlfr_ph_F (5'-
186 TACAGACCAGTCCGAGCAAAA -3'), Chlfr_ph_R (5'- TTGACGACGCCAATGTTCTCT -3') and probe Chlfr_ph_P (5'-

187 FAM- TCCCTTAGGCGTGGCATCCTTCAACT -TAMRA-3') targeting the RNA polymerase sigma-54 factor gene *rpoN*
188 were used in a TaqMan assay. For another new species comprising isolates 15-2067_O50 and 15-2067_O99, primers
189 Chlfr_vo_F (5'- TCCTGCTCCTTGAGAAAAGCT -3'), Chlfr_vo_R (5'- CATTGTCCTTCATGCTCTCGT -3') and probe
190 Chlfr_vo_P (5'- FAM-TCCTCTAAGACCGCCATTGCCTGATGT-TAMRA-3') targeting the *rpoN* gene of this species
191 were designed for the species-specific TaqMan assay. Mix concentrations and cycle parameters are the same as
192 described above except the annealing/elongation step, which is performed at 95°C for 3s and 60°C for 30 s. The
193 specificity of each assay was evaluated on 30 samples of different *Chlamydiaceae* DNA (Table S1).

194

195 Phylogenetic analysis

196 A similarity matrix of the 16S and 23S rRNA genes was calculated using a pairwise alignment with a Jukes and Cantor
197 correction of the three genomes and other established species. Moreover, nine phylogenetic markers previously
198 described [36] were extracted from the 11 *Chlamydiaceae* reference sequences, from *Candidatus C. ibidis*, from the
199 three isolates 14-2711_R47, 15-2067_O50 and 15-2067_O99 and from seven non-*Chlamydiaceae*. Amino acid
200 sequences were aligned with MAFFT v7 [19] and concatenated to build a reference phylogeny using RAXML v8 [45],
201 with the LG+G+I model and 100 bootstrap replicates. Pairwise amino acid sequence identities were calculated based
202 on the MAFFT alignment using Geneious v10 (www.geneious.com).

203 Plasmids of 11 *Chlamydiaceae* reference strains and the three new flamingo isolates were aligned using MUSCLE
204 v3.8.31 [8], and a phylogenetic tree was built using RAXML v8 with the GTRGAMMA model and 100 bootstrap
205 replicates.

206 The plasmids of the three new strains were annotated manually by identifying the ORFs using Geneious v10. Each
207 ORF was searched (BLASTX) against the NCBI non-redundant protein sequence database for annotation. Linear
208 comparison of plasmids was constructed using Easyfig v2.2.3 [47] with the *C. psittaci* plasmid pCps6BC serving as a
209 reference.

210

211 Pairwise comparison of genome sequences, percentage of conserved proteins (POCP), and average amino acid
212 identity (AAI).

213 The parameters of the tetranucleotide signature frequency correlation coefficient (tetranucleotide regression) and
214 average nucleotide identity (ANIb) were determined as described previously [38] using the program JSpecies v1.2.1
215 with default parameters. The percentage of conserved proteins (POCP) was calculated using a Ruby implementation of
216 the formula given by Qin [37] (<https://github.com/hoelzer/pocp>) and the average amino acid identity (AAI) was
217 calculated using the MiGA program [39].

218

219 Additional long-read-based analysis of major outer membrane protein (MOMP) and in-silico assessment

220 Nanopore long reads spanning the region of interest with multiple copies of *ompA*-like genes were visualized from the
221 long read mapped to the hybrid assembly using the matplotlib library [16]. The underlying python script can be found in
222 the OSF repository (<https://osf.io/zwgc9/>). Besides, we performed draft assemblies for all three flamingo strains with
223 the ONT long-reads only using Flye [20] to reduce errors possibly induced by an initial short-read graph as used by

224 Unicycler. First, the longest ONT reads were selected with Filtlong v0.2.0 (<https://github.com/rrwick/Filtlong>) to achieve
225 a coverage of approximately 100X per strain and subsequently assembled with Flye using the parameters —plasmids,
226 —meta, and a genome size of 1 Mbp. The draft assemblies were then polished in one round of Racon v1.4.7 [50] using
227 minimap2 v2.17 to align the long reads followed by a final polishing with Medaka v1.0.3
228 (<https://github.com/nanoporetech/medaka>) using the r941_min_high_g360 model. Sequences identified as MOMP
229 based on the Prokka annotation were extracted for each of the new *Chlamydiifrater* isolates. Subsequently, these
230 sequences were aligned to a full-length reference MOMP of *Chlamydia pneumoniae* CWL029 (AE001363.1) using
231 Mafft v7.402 with default parameters. The alignments were visualized using Clustalx 2.1.

232
233
234

235 Results

236

237 Detection of atypical chlamydiae during routine examination of captive flamingos

238 In 2011, a flock of 15 flamingos (*Phoenicopterus chilensis*) was acquired by the Bioparc zoo and kept in quarantine
239 until diagnosed negative for avian chlamydiosis and other diseases. Cloacal swabs were taken from these birds and,
240 using a *Chlamydiaceae*-specific PCR, three samples tested positive (id 11-261) in a private diagnostic laboratory. In
241 the National Reference Laboratory, positivity for the family *Chlamydiaceae* was confirmed, but these samples tested
242 negative for *C. psittaci*, *C. avium*, *C. gallinacea* and *Candidatus C. ibidis* using species-specific real-time PCR assays.
243 While still in quarantine, all 15 birds were re-sampled (id 11-324) and 10 of them tested positive for *Chlamydiaceae*,
244 with a mean Cq value of 36.8 (Table 1). Using BLASTN search, the 16S rRNA gene sequence of one of the high-titer
245 samples (11-261_B096) showed highest identity (97%) to an uncultured *Chlamydiales* bacterium (clone 122) detected
246 from a seabird. Unfortunately, no isolate was recovered from these sample sets. In 2014, another 37 captive flamingos
247 (*Phoenicopterus roseus*) from the Paris Zoo (id 14-1167) were sampled, with only one detected as a weak positive
248 (Table 1).

249

250 Detection of atypical chlamydiae in wild flamingo chicks

251 In France, catches of juvenile flamingos for banding are organized on a yearly basis. Between 2012 and 2015, 404
252 birds were submitted to cloacal swabbing. PCR examination of the samples showed 30.9% (125/404) of the birds
253 harboring chlamydiae in their digestive tract (Table 1). When re-tested for *C. psittaci*, *C. avium*, *C. gallinacea* and
254 *Candidatus C. ibidis*, none of the samples proved positive. From 5 PCR-positive samples subjected to cell culture,
255 chlamydiae were successfully cultured in three of them: 14-2711_R47 (from Salins d'Aigues Mortes, 2014), 15-
256 2067_O50 (Etang du Fangassier, 2015) and 15-2067_O99 (Etang du Fangassier, 2015).

257

258 Molecular characterization of the *Chlamydiaceae* detected in flamingos

259 To determine the genealogical position of the chlamydiae encountered in the flamingos, nearly complete 16S rRNA
260 gene sequences (964 to 1546 nt) were obtained from eight chlamydia-positive samples. Later, complete 16S and 23S
261 rRNA sequences of the three isolates 14-2711_R47, 15-2067_O50 and 15-2067_O99 were extracted from whole-

262 genome sequences. The phylogenetic tree inferred from multiple alignments of the 16S rRNA gene sequences shows
263 the flamingo isolates forming a distinct cluster that is clearly separated from the established *Chlamydia* spp., but still
264 within the family *Chlamydiaceae* (Figure 1). Numerical analysis revealed that the three flamingo isolates shared less
265 than 96% 16S rRNA identity with the other chlamydial species (Table 2), with the closest genetic relatedness found to
266 *Candidatus C. ibidis*, i.e. 95.64% identity for isolate 14-2711_R47 and 95.57% for isolates 15-2067_O50 and 15-
267 2067_O99. Comparison of 23S rRNA gene sequences revealed that the most closely related species was also
268 *Candidatus C. ibidis* with identities of 95.39% and 95.60% (Table 2). Interestingly, ribosomal sequences of isolates 15-
269 2067_O50 and 15-2067_O99 proved identical, but different from those of 14-2711_R47, with 16S and 23S rRNA
270 identity values of 99.55% and 99.32%, respectively.

271

272 **Genome data and comparison with other species of the *Chlamydiaceae***

273 The three new flamingo isolates were subjected to Illumina and Nanopore sequencing. *De novo* hybrid assembly of
274 raw reads produced a single scaffold representing the entire chromosome for each of the three isolates. Assembly
275 parameters, such as percentage of mapped reads, error rate and coverage of chromosomes and plasmids are given in
276 Table S2.

277 Basic parameters of whole-genome sequences as compiled in Table 3 are similar to those of the established
278 *Chlamydia* spp. In particular, the number of CDS identified in isolates 14-2711_R47, 15-2067_O50 and 15-2067_O99
279 are 981, 980, and 983, respectively; G+C values are slightly above 40%, and only one rRNA operon and one plasmid
280 were found. Genome sizes of the flamingo isolates are between 1,182 and 1,195 kbp and thus slightly larger than
281 *Chlamydia* spp. genomes, except *C. pneumoniae* (1,225 kbp).

282 The values of average nucleotide identity (ANIb) and tetranucleotide signature correlation index (TETRA) presented in
283 Table 4 confirm that the flamingo isolates are genetically different from currently known *Chlamydia* spp. For instance,
284 isolate 15-2067_O50 showed ANIb values ranging from 65.70 (*C. pecorum* E58^T) to 66.20 (*Cand. C. ibidis* 10-1398/6),
285 and TETRA indexes from 0.6294 (*C. pneumoniae* TW-183^T) to 0.8379 (*Cand. C. ibidis* 10-1398/6). Typical values
286 between members of the genus *Chlamydia* are in the range from 66.76 (*C. suis* SWA-2 vs. *C. pecorum* E58^T) to 92.58
287 (*C. psittaci* 6BC^T vs. *C. abortus* S26/3) and 0.7106 (*Cand. C. ibidis* 10-1398/6 vs. *C. pneumoniae* TW-183^T) to 0.9864
288 (*C. gallinacea* 08-1274/3^T vs. *C. avium* 10DC88^T).

289 As this distant relatedness raised the question of whether the flamingo strains could belong to a distinct genus, further
290 analyses were performed. The percentage of conserved proteins (POCP) ranged from 66.55 (15-2067_O99 vs. *C.*
291 *pneumoniae* TW-183^T) to 71.29 (14-2711_R47 vs. *C. pecorum* E58^T) (Table 5, upper triangle), confirming the previous
292 findings on the genetically distant position of the three isolates compared to currently known *Chlamydia* spp., as well as
293 near genetic identity of isolates 15-2067_O50 and 15-2067_O99. Another parameter tested to assess the taxonomic
294 rank of these new isolates was the average amino acid identity (AAI). Previous findings [21, 27] indicated that,
295 preponderantly, organisms grouped at genus level share between 60 and 80% AAI and are clearly distinguishable from
296 those grouped at species level, which display more than 85% AAI. As can be seen from Table 5, lower triangle, isolate
297 14-2711_R47 compared to isolates 15-2067_O50 and 15-2067_O99 shows 80.45 and 80.51% AAI, respectively. The

298 three isolates share less than 60% AAI with the members of genus *Chlamydia*, while closely related isolates 15-
299 2067_O50 and 15-2067_O99 are at 99.58% AAI.

300 These data are also supported by genome analysis using a classification scheme recently proposed for members of
301 the order *Chlamydiales* [36]. In this scheme, the percentage of amino acid sequence identity is calculated for nine
302 conserved gene products (RpoN, FtsK, PepF, Adk, HemL, Dna, SucA, protein_325 and FabI) extracted from the
303 proteome sequences of the flamingo isolates and the other 11 chlamydial species, plus *Candidatus* C. ibidis and seven
304 non-*Chlamydiaceae*. The phylogenetic tree based on these nine proteins in **Figure 2** clearly indicates that the flamingo
305 isolates are clustered in a single clade (bootstrap value 100%) that is clearly separated from the main clade harboring
306 all known members of the genus *Chlamydia*. Within the flamingo clade, isolate 14-2711_R47 forms its own branch
307 apart from 15-2067_O50 and 15-2067_O99 (bootstrap value 100%). **Pairwise amino acid identity of the three flamingo**
308 **isolates (Table S3) shows that they belong to the family Chlamydiaceae with values above cut-off for the 16S rRNA**
309 **and 23S rRNA, but clearly outside the genus Chlamydia, as indicated by low sequence homologies of the protein_325**
310 **(56.75 to 61.91%), DnaA (58.70 to 62.17%), SucA (55.20 to 59.55%) and FabL (74.58 to 82.27%) proteins with their**
311 **homologs in the genus Chlamydia.** In contrast, isolate 14-2711_R47 shares low sequence similarities of the RpoN,
312 PepF, Adk and FtsK proteins with the isolates 15-2067_O50 and 15-2067_O99, thus suggesting that they represent
313 different species. Finally, the two nearly identical isolates 15-2067_O50 and 15-2067_O99 share 100% amino acid
314 identity for most of the taxonomically relevant proteins, except for SucA and FtsK (99.79% and 99.76% identity,
315 respectively), indicating that they belong to the same species.

316 Further comparative genome analysis of the flamingo isolates and type strains of 11 members of the genus *Chlamydia*
317 plus *Candidatus* C. ibidis included the use of UpSetR, a software package for visualization of shared and unique
318 genomic items (**Figure 3**). While the new strains share a core genome of 756 genes with the species of the genus
319 *Chlamydia*, a set of 110 genes, roughly 11 % of their coding capacity, is exclusively encountered in the three flamingo
320 strains. In addition, 29 genes are present only in strains 15-2067_O50 and 15-2067_O99, and another 31 genes are
321 unique to strain 14-2711_R47. This illustrates the separate genetic position of the three flamingo isolates apart from
322 the genus *Chlamydia*, and also the close relatedness of strains 15-2067_O50 and 15-2067_O99 as well as their
323 distinction from 14-2711_R47 within the new entity.

324 Moreover, plasmids sized 6,358 bp, 6,320 bp and 6,319 bp were found in strains 14-2711_R47, 15-2065_O50 and 15-
325 2067_O99, respectively. The phylogenetic tree in **Figure S1** shows a distinct clade containing the flamingo isolates at
326 a bootstrap value of 100. **Comparison of linearized plasmids revealed completely conserved synteny in all three**
327 **flamingo strains but in comparison with the C. psittaci plasmid, the integrase pGP7-D gene is missing (Figure S2).**

328 All flamingo strains possess one copy of a full-length *ompA* with 56.10 to 58.82 % nucleotide sequence similarity
329 (53.47 to 55.86 % aa similarity) to *C. pneumoniae ompA* as its closest relative (**Tables S4 and S5**). Among *Chlamydia*
330 spp. these minimal similarity values are at 64.02% (*C. gallinacea ompA* vs. *C. suis*) and, at aa level, 62.44% (*C.*
331 *pecorum* vs. *C. suis*). In addition, all flamingo strain genomes were found to harbor a group of truncated OmpA-like
332 porins (n=15, 5 or 9) that are arranged in two clusters and lack the N-terminal conserved domain of the chlamydial
333 major outer membrane proteins (MOMPs). Their sequences are variable, but they share the general OmpA/MOMP

334 structure of alternating variable and conserved domains (**Figure S3**). Among these items, nucleotide sequence
335 similarities to *C. pneumoniae ompA* are typically 40-55% (35 to 50% at aa level), while individual OmpA-like proteins of
336 the same strain share less than 80% sequence similarity (data not shown). To rule out the possibility of truncated
337 *ompA*-like genes being due to assembly artifacts, we mapped the ONT read data to the corresponding hybrid
338 assemblies using minimap2 with parameter settings specific for ONT long-reads. **Figure S4** provides a schematic
339 overview of selected Nanopore long reads spanning the region of interest with multiple copies of *ompA*-like genes,
340 exemplary for strain 15-2067_O99 and thus providing strong evidence for the correctness of the assembly. In addition,
341 we performed draft assemblies only based on the ONT long-reads to avoid misassemblies derived from an initial short-
342 read graph (as used in the hybrid-approach in Unicycler). As in the hybrid-assembly approach, we also observed
343 multiple *ompA*-like genes in these long-read-based assemblies (data not shown).

344

345 **Morphological features of flamingo isolate 14-2711 R47**

346 In BGM-cell culture samples collected at 24h p.i., no chlamydial structures were detected in TEM images. At 48h p.i.
347 inclusions were present in a few cells. Infected cells were either of elongated and slender or round shape (**Figure 4A**).
348 The cytoplasm of infected cells was replaced with inclusions that were incompletely separated by thin strands of
349 cytoplasm (**Figure 4A, B**). These may either represent multiple inclusions or a large convoluted inclusion. Aggregates
350 of small round electron-dense elementary bodies (EBs, 190-290 nm), aggregates of large, pleomorphic or elongated,
351 less electron-dense reticulate bodies (RBs, 580-780 x 190-580 nm), and mixed aggregates were observed in the same
352 cell (**Figure 4A, B**). Overall, EBs predominated at 48h p.i., but there were a few cells with multiple inclusions containing
353 predominantly RBs (**Figure 4C**). Intermediate bodies (IB) with a central electron-dense core, as seen in *Chlamydia*
354 spp., were not detected. In some cells, vacuoles with flocculate material were located adjacent to inclusions.
355 Comparable vacuoles were also present in non-infected cells. At 72h p.i., inclusions were present in numerous cells.
356 Most infected cells were slender and elongated. The morphology of inclusions was similar to that described at 48h p.i.
357 and both cells with predominantly EBs as well as with RBs were seen (**Figure 4D**). EBs were round, and RBs
358 appeared elongated or pleomorphic (**Figure 4D, E, F**). Vacuolization of cells was more pronounced than at 48h p.i.
359 (**Figure 4E**). In many cells, areas of debris and membrane whirls were present between aggregates of EBs and RBs
360 (**Figure 4E, F**).

361

362 **Morphological features of chlamydial isolate 15-2067 O50**

363 As above, chlamydial inclusions were not seen in BGM-cell culture at 24h p.i. At 48h p.i., inclusions appeared in a few
364 cells that were often round and contained large vacuoles (**Figure 5A**). A few bacteria were present within those
365 vacuoles and many spread throughout the remaining cytoplasm (**Figure 5A**). Whirls of membranes that might
366 represent disintegrated bacteria (empty shells) or cellular organelles were identified in some of the cells (**Figure 5B**).
367 EBs predominated at that stage (**Figure 5C**), but there were also a few cells containing predominantly RBs (**Figure**
368 **5D**). EBs were round to crescent-shaped and had a wide periplasmic space (**Figure 5C**), RBs were pleomorphic or
369 elongated (**Figure 5B, D**). Again, no intermediate bodies (IB) with a central electron-dense core were found. At 72h
370 p.i., chlamydial bodies were present in numerous cells that were often rounded. Numerous EBs and RBs were evenly

371 distributed throughout the cytoplasm (Figure 5E). Small groups or single bacteria were separated by thin strands of
372 cytoplasm (Figure 5F). EBs (190 – 290 nm) were of variable electron density. Wide periplasmic spaces separated cell
373 wall and cytoplasm of EBs. EBs displayed a highly variable morphology ranging from round to crescent-shaped to
374 irregular. RBs (580 -780 nm x 190 – 390 nm) were less electron dense than EBs and pleomorphic or elongated (Figure
375 5F). In many cells, vacuoles of various sizes were observed in the cytoplasm.

376

377 **Detection assays for the new genus and the new species**

378 A specific real-time PCR assay for the new genus comprising the flamingo strains described in this study was
379 developed based on the 16S rRNA gene locus (see Materials and Methods). This locus is well characterized for
380 organisms of the order *Chlamydiales* and contains specific targets for most, if not all, genera. To validate the assay,
381 DNA aliquots of *C. abortus*, *C. avium*, *C. caviae*, *C. gallinacea*, *C. felis*, *Candidatus C. ibidis*, *C. muridarum*, *C. suis*, *C.*
382 *trachomatis* *C. pecorum* and *C. psittaci* strains and all samples positive in *Chlamydiaceae*-specific real-time PCR of
383 this study were run using the present protocol. The experiments revealed 115 positive samples out of 136
384 *Chlamydiaceae*-PCR positives. Those 21 samples classified as negative (below detection limit) still had Cq values
385 ranging from 37.10 to 39.97 (Table1).

386 The same DNA aliquots were tested against newly developed real-time PCRs to detect the new species comprising a)
387 strain 14-2711_R47^T or b) strains 15-2065_O50^T and 15-2067_O99, respectively. These two assays proved more
388 sensitive than the genus-specific PCR as we detected among the 21 negatives four more positive samples for the
389 former and two for the latter new species. Interestingly, four samples from the preliminary investigation showed a
390 positive signal for both species, possibly indicating dual infection.

391 The test results yielded detection rates of 9.6% (13/136) and 86.8% (118/136) for a) and b), respectively (Table 1).

392

393

394 **Discussion**

395

396 The present study was undertaken as part of the routine precautionary biosafety measures to protect birds in a large
397 aviary. To reduce the risk of infection(s), it is mandatory for new entries to undergo appropriate diagnostic testing and
398 quarantine. When a group of quarantined Chilean Flamingos destined to be integrated into a large aviary complex was
399 examined, *Chlamydiaceae*-positive individuals were identified. These initial findings prompted further samplings in this
400 group, as well as in another group of captive Greater Flamingos from another zoo and in wild Greater Flamingos.
401 Altogether, 52 captives and 404 wild birds were sampled yielding a PCR-based *Chlamydiaceae* prevalence of 21.1 and
402 30.9%, respectively. Notably, the usual avian chlamydial species, i.e. *C. psittaci*, *C. avium* and *C. gallinacea* were
403 absent in these birds. In further testing of *Chlamydiaceae*-positive samples, newly developed real-time PCR assays
404 revealed detection rates of 86.8% for a new species represented by flamingo isolates strains 15-2067_O50 and 15-
405 2067_O99 and 9.6% for another new species comprising strain 14-2711_R47.

406 All chlamydia-positive flamingos in the present study were visually found to be in good health, independently of their
407 habitat (captive or wild). Therefore, we hypothesize that chlamydiae of the new taxa behave as (gastro-intestinal)
408 commensal bacteria in healthy birds. Further investigations are required to find out whether they bear the potential of

409 causing disease under specific circumstances as is the case with other avian members of the *Chlamydiaceae*. Future
410 studies will also have to show whether flamingos are the only natural host of these new agents.

411

412 The 16S rRNA sequences from captive and wild flamingos were highly similar to a sequence detected in a seabird that
413 had been caught in the Behring Sea (Uncultured *Chlamydiales* bacterium clone 122, from a glaucous-winged gull [3]),
414 as well as to sequences from seabirds sampled from common murre (12-3998_AO053 and 12-3998_AO018) and a
415 European herring gull (12-1761_J072) in France [1]. Closely related non-classified *Chlamydiaceae* were also
416 sporadically detected in two flocks of farmed ducks [15], thus suggesting that, even though encountered only
417 temporarily at one farm, these chlamydiae are not confined to seabirds. All 16S rRNA sequences from cultured and
418 uncultured flamingo specimens formed a distinct clade outside the genus *Chlamydia*, but within the family
419 *Chlamydiaceae*. The above-mentioned Uncultured *Chlamydiales* bacterium clone 122 and other seabird specimens
420 proved to be their closest relative (**Figure 1**).

421 According to currently accepted rules, "sequence identity of 94.5% or lower for two 16S rRNA genes is strong evidence
422 for distinct genera" [53], this suggests that the present flamingo isolates should be assigned to a new genus. In this
423 context, POCP has become a widely accepted parameter for delimiting genus boundaries in prokaryotic genome-
424 based taxonomy. It involves comparison of proteome sequences including all proteins of a species or strain, and a
425 POCP value of 50% was proposed as a genus boundary [37]. This means that two strains would share less than half of
426 their protein repertoires if they belong to different genera. Here, POCP values determined for the flamingo isolates
427 proved considerably higher than the 50% cut-off (**Table 5**), thus impeding formal introduction of a separate genus in
428 the family *Chlamydiaceae*. However, this parameter has been used by microbiologists and taxonomers for only five
429 years, so that there is little or no experience on the possibility of a more flexible use of the cut-off in particular cases. At
430 the same time, the average amino acid identity (AAI) has been used since 2005 [21] and is considered a robust
431 measure of genetic relatedness between two strains. The values ranging from 55.30 to 56.54% for the flamingo strains
432 are clearly below the recommended level of approximately 70% for genus boundaries, thus supporting the idea of a
433 separate genus.

434

435 TEM revealed that both isolates 14-2711_R47 and 15-2067_O50 replicated intracellularly and developed EBs and
436 RBs, which is characteristic for the biphasic replication cycle of *Chlamydiaceae*. Notably, inclusion morphology differed
437 from other members of the genus *Chlamydia*, e.g. *C. psittaci*, *C. abortus*, *C. pecorum*, *C. trachomatis*, *C. avium*, or *C.*
438 *gallinacea*. In most late infected host cells, aggregates of bacteria separated by variably thick strands of cytoplasm
439 filled the entire cell. This was interpreted as multiple inclusions fusing and forming large branching inclusions. The
440 inclusion morphology could reflect differences in intracellular trafficking [11]. In addition, no intermediate bodies (IBs)
441 with the characteristic morphology described for the genus *Chlamydia* were observed alongside EBs and RBs in both
442 type strains. Since differences in inclusion and EB/RB morphology were reported between the various families of
443 *Chlamydiales* [2, 5, 7, 12-14, 26], between species of *Chlamydia* [33], and even between isolates of the same species
444 [31], the detection of such morphological distinctions between flamingo strains and other *Chlamydiaceae* is not entirely

445 surprising. Within the species of *C. pneumoniae*, wide periplasmic spaces were detected only in the Koala strain
446 LPCoLN, but not the human isolate AR39 [31].

447 Other aspects to be considered when interpreting the particular morphology of strains 14-2711_R47 and 15-2067_O50
448 are treatment prior to fixation [29] and influence by the preparation. Although standardized methods were used, an
449 effect cannot be excluded, since conventional electron microscopy relies on fixation and embedding of samples into
450 resin. Different osmolarities of fixatives and buffers caused changes in morphology, particularly in the family
451 *Parachlamydiaceae*, with higher osmolarity resulting in shrunken, star-shaped or crescent-shaped EBs [40]. This was
452 interpreted as an indication of a distinct membrane composition or intracellular structure [40]. Crescent-shaped EBs
453 and wide periplasmic spaces were observed in isolate 15-2067_O50 only, although both isolates were treated under
454 identical conditions. Thus, potential differences between the type strains should be explored further. Interestingly, only
455 the metabolically inactive EBs were affected. Whole-cell cryo-electron tomography revealed polarization of the type 3
456 secretion system on EBs during distinct stages of their development [32]. This may increase their susceptibility to
457 deformation. Finally, both strains were cultured in BGM cells, which may not be as optimal as cells from their natural
458 host, the flamingo. Conceivably, this could also have contributed to the differences to classical chlamydial morphology
459 observed here. The influence of culture conditions, e.g. temperature, on inclusion morphology has been demonstrated
460 for various veterinary and human chlamydial species, especially those causing infections in reptiles [33]. Some of the
461 vacuoles and membrane whirls observed in isolates 14-2711_R47 and 15-2067_O50 may represent remnants of
462 degraded bacteria.

463

464 Concerning species assignment, the current recommendation for the genomic circumscription of species by means of
465 pairwise comparison prescribes ANI higher than 96% and TETRA values above 0.999 [38]. While the present isolates
466 15-2076_O50 and 15-2076_O99 seem to belong to the same species (ANI 99.46 and TETRA 0.9997; **Table 4**), isolate
467 14-2711_R47 shows distinct parameters (e.g. 75.53 and 0.9460 to strain 15-2067_O50), the latter indicating that it
468 probably represents a different species. This notion is confirmed by POCP values of 99.85 for the closely related
469 strains, as compared to 95.41 (14-2711_R47 vs. 15-2076_O50) and 95.26 (14-2711_R47 vs. 15-2076_O99),
470 respectively (**Table 5**). POCP values between different chlamydial species can be as high as 98.64 (*C. psittaci* vs. *C.*
471 *abortus*) and 96.65 (*C. avium* vs. *C. gallinacea*), although it should be noted that the parameter is not regarded valid for
472 species circumscription.

473 The present data from comparative genomic analysis clearly illustrate i) the distinct genetic position of the flamingo
474 strains compared to members of the genus *Chlamydia* and ii) the nearly identical genome composition of new strains
475 15-2076_O50 and 15-2076_O99 compared to strain 14-2711_R47. While the flamingo strains share 756 homologous
476 CDS, i.e. roughly three-fourths of their genomes, with 12 members of the genus *Chlamydia*, there is also a
477 considerable portion of 176 CDS occurring only in these three isolates (**Figure 3**). Besides, the genome of isolate 14-
478 2711_R47 harbors 31 unique genes, which illustrates its distinction from the other two flamingo isolates. Based on
479 shared genomic items, no particularly close relationship to certain individual *Chlamydia* spp. can be derived from these
480 data, which is in accordance with the phylogenetic tree in **Figure 2**.

481 Analysis of a validated panel of nine conserved taxonomically relevant proteins was also fully in line with the genome
482 data (**Figure 2**), thus suggesting assignment of the three isolates to two different species.

483 In addition, the new strains described here proved distinct from *Chlamydia* spp. by the presence of multiple truncated
484 sequences of *ompA*-like genes besides a 'classical' *ompA*. As this is a completely new finding, possible functions of the
485 truncated proteins, if expressed and translated, have yet to be elucidated. It is conceivable that they could enhance
486 functions known from the main outer membrane protein encoded by *ompA*, i.e. cytoadhesion and immunogenicity, as
487 well as contribute to the bacterium's outer membrane stability.

488
489 Compilation of the main characteristics and distinctive features between the present flamingo strains and the
490 organisms classified in the genus *Chlamydia* (**Table 6**) clearly illustrates the separate taxonomic position of the former.

491 Therefore,

492 we propose the introduction of a new genus *Chlamydiifrater* gen. nov. with two new species, i.e. *Chlamydiifrater*
493 *phoenicopteri* sp. nov. with type strain 14-2711_R47^T and *Chlamydiifrater volucris* sp. nov., with strains 15-2067_O50^T
494 (type strain) and 15-2067_O99. The formal proposal of the new genus and species is given in the protologue (**Table 7**).

495 TABLES AND FIGURES

496 **Table 1:** Summary of test results of flamingo specimens involving four different real-time PCR assays with specificities
497 for the family *Chlamydiaceae*, the proposed new genus and the two new species.

498 **Table 2:** Pairwise sequence identity values for complete 16S (lower triangle) and 23S (upper triangle) ribosomal RNA
499 gene loci of the flamingo isolates and representative strains of 12 species of the genus *Chlamydia* (extracted from
500 whole-genome sequences).

501 **Table 3:** Basic features of *de novo* sequenced flamingo strain genomes and representative strains of 12
502 *Chlamydia* spp.

503 **Table 4:** Pairwise comparison of genome sequences of the new flamingo strains and 12 representative strains of
504 *Chlamydia* spp. based on average nucleotide identity (ANI; lower triangle) and tetranucleotide signature correlation
505 index (TETRA; upper triangle).

506 **Table 5:** Average amino acid identity (AAI; lower triangle) and percentage of conserved proteins (POCP; upper
507 triangle) of the new flamingo strains and 12 representative strains of *Chlamydia* spp.

508 **Table 6:** Characteristic traits and features of *Chlamydiifrater* gen.nov. in comparison to the genus *Chlamydia*

509 **Table 7:** Protologue on the proposal of *Chlamydiifrater* gen. nov. and *Chlamydiifrater phoenicopteri* sp. nov. and
510 *Chlamydiifrater volucris* sp. nov.

511

512 **Figure 1:** Phylogenetic reconstruction based on almost complete 16S rRNA sequences of 12 field samples (marked
513 with an *), complete 16S rRNA sequences of the three flamingo isolates and type or reference strains of established
514 *Chlamydia* spp. *Simkania negevensis* was used as an outgroup. The tree has been reconstructed using RAxML v8.
515 Bootstrap values indicate stability of the branches based on 1000 replicates. The lower bar indicates the percentage of
516 divergence.

517 **Figure 2:** Phylogenetic tree of the genus *Chlamydia* and the new *Chlamydiifrater* isolates. The phylogeny was
518 reconstructed based on the concatenated alignment of DnaA, SucA, protein_325, FabI, RopN, FtsK, PepF, Adk and
519 HemL [36]. Bootstrap values are shown as percentages. The scale bar indicates the number of amino acid
520 substitutions per site. The taxa from outside the family *Chlamydiaceae* have been included for comparison.

521 **Figure 3:** UpSet bar diagram showing the numbers of genes shared by various groups of chlamydial strains involved in
522 this study. Calculations are based on whole-genome sequences of the flamingo strains and type or reference strains of
523 12 members of the genus *Chlamydia*. Orthologous groups were calculated using the RIBAP pipeline and used as input
524 for UpSetR. Cfph = *Chlamydiifrater phoenicopteri*, Cfvo = *Chlamydiifrater volucris*, Cab = *Chlamydia abortus*, Cps =
525 *Chlamydia psittaci*, Cca = *Chlamydia caviae*, Cfe = *Chlamydia felis*, Cav = *Chlamydia avium*, Cga = *Chlamydia*
526 *gallinaceae*, Cib = *Candidatus Chlamydia ibidis*, Cpe = *Chlamydia pecorum*, Cpn = *Chlamydia pneumoniae*, Csu =
527 *Chlamydia suis*, Cmu = *Chlamydia muridarum*, Ctr = *Chlamydia trachomatis*.

528 **Figure 4:** BGM cells infected with *Chlamydiifrater phoenicopteri* strain 14-2711_R47^T for 48 hours (A, B, C) and 72
529 hours (D, E, F). (A) Infected cells are elongated and slender. Most of the cytoplasm is filled by either multiple confluent
530 chlamydial inclusions or one large branching inclusion. EB predominate. (B) Higher magnification of (A): thin strands of
531 cytoplasm (dotted lines, examples) extend between aggregates of bacteria. Some aggregates consist predominantly of
532 EBs (E), some of RBs (R), and some of both EBs and RBs (M). (C) Cell with multiple inclusions (outlined by dotted

533 lines) that contain predominantly RBs. RBs are pleomorphic. (D). Adjacent infected cells may contain predominantly
534 RBs (1) or EBs (2). Both cells are elongated. The cytoplasm is replaced with numerous bacteria. Delicate strands of
535 cytoplasm separate aggregates of bacteria. (E) Vacuole (V) with flocculate material adjacent to inclusions with EBs (E)
536 and RBs (R). Some RBs are particularly slender and elongated (arrowheads). The cytoplasm between inclusions
537 contains small vacuoles, membrane whirls and cellular debris (D). (F) Higher magnification of EBs (E, examples), RBs
538 (R, examples) and cellular debris (D) in an infected cell. RBs are elongated (L) and pleomorphic (P). Bar in A = 2 μm ,
539 bars in B-F = 1 μm .

540 **Figure 5:** BGM cells infected with *Chlamydiafrater volucris* strain 15-2067_O50^T for 48 hours (A, B, C) and 72 hours (D,
541 E). (A) Infected cell with large vacuoles (V) containing a few EBs and with numerous bacteria throughout the
542 cytoplasm.
543 (B) Inclusions with predominantly EBs and few RBs (R). Some EBs are crescent-shaped (arrowheads, examples) and
544 some have wide periplasmic spaces (arrows, examples). (C) RBs predominate in a few cells. RBs are separated by
545 strands of cytoplasm. RBs are pleomorphic to elongated (L). (D) Higher magnification of EBs (E), RBs (R) and whirls of
546 membranes (arrows, examples) within a cell. (E) Confluent inclusions fill the cytoplasm of an infected cell. Both EBs
547 and RBs are present. (F) Higher magnification of EBs and RBs in an infected cell. EBs are round to crescent-shaped
548 (arrowheads, examples) and have a wide periplasmic space (arrows, examples). RBs are pleomorphic (P) or elongated
549 (L). Bacterial aggregates are separated by delicate strands of cytoplasm (dotted lines, examples). Bars in A, E = 2 μm ,
550 bars in B, C, D, F = 1 μm .

551
552

553 **Acknowledgments**

554 Sampling of flamingos was performed in collaboration with the research center Tour du Valat in the framework of
555 annual catches for ring identification organized by Arnaud Béchet. We are particularly thankful to Olivier Yvernogean
556 (Genindexe) for sharing DNA samples from flamingos for initial analysis. The authors would also like to thank Lisa Wolf
557 and Jana Jagiela for excellent preparation of samples for electron microscopy and Wolfram Maginot for his excellent
558 photographic assistance. Martin Hölzer appreciates the support of the Joachim Herz Foundation by the add-on
559 fellowship for interdisciplinary life science.

560

561 **Conflict of interest statement**

562 None of the authors has a financial or personal relationship with other people or organisations that could
563 inappropriately influence or bias this paper

564

565 **References**

566

567 [1] Aaziz, R., Gourlay, P., Vorimore, F., Sachse, K., Siarkou, V.I., Laroucau, K. (2015).
568 Chlamydiaceae in North Atlantic Seabirds Admitted to a Wildlife Rescue Center in
569 Western France. *Appl Environ Microbiol.* 81 4581-4590.

570 [2] Amann, R., Springer, N., Schonhuber, W., Ludwig, W., Schmid, E.N., Muller, K.D.,
571 Michel, R. (1997). Obligate intracellular bacterial parasites of acanthamoebae related to
572 Chlamydia spp. Appl Environ Microbiol. 63 115-121.

573 [3] Christerson, L., Blomqvist, M., Grannas, K., Thollesson, M., Laroucau, K.,
574 Waldenstrom, J., Eliasson, I., Olsen, B., Herrmann, B. (2010). A novel Chlamydiaceae-
575 like bacterium found in faecal specimens from sea birds from the Bering Sea. Environ
576 Microbiol Rep. 2 605-610.

577 [4] Conway, J.R., Lex, A., Gehlenborg, N. (2017). UpSetR: an R package for the
578 visualization of intersecting sets and their properties. Bioinformatics. 33 2938-2940.

579 [5] Corsaro, D., Thomas, V., Goy, G., Venditti, D., Radek, R., Greub, G. (2007).
580 '*Candidatus Rhabdochlamydia crassificans*', an intracellular bacterial pathogen of the
581 cockroach *Blatta orientalis* (Insecta: *Blattodea*). Syst Appl Microbiol. 30 221-228.

582 [6] Di Tommaso, P., Chatzou, M., Floden, E.W., Barja, P.P., Palumbo, E., Notredame, C.
583 (2017). Nextflow enables reproducible computational workflows. Nat Biotechnol. 35
584 316-319.

585 [7] Draghi, A., 2nd, Popov, V.L., Kahl, M.M., Stanton, J.B., Brown, C.C., Tsongalis,
586 G.J., West, A.B., Frasca, S., Jr. (2004). Characterization of "*Candidatus piscichlamydia*
587 *salmonis*" (order *Chlamydiales*), a chlamydia-like bacterium associated with
588 epitheliocystis in farmed Atlantic salmon (*Salmo salar*). J Clin Microbiol. 42 5286-5297.

589 [8] Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced
590 time and space complexity. BMC Bioinformatics. 5 113.

591 [9] Ehricht, R., Slickers, P., Goellner, S., Hotzel, H., Sachse, K. (2006). Optimized DNA
592 microarray assay allows detection and genotyping of single PCR-amplifiable target
593 copies. Mol Cell Probes. 20 60-63.

594 [10] Garcia-Alcalde, F., Okonechnikov, K., Carbonell, J., Cruz, L.M., Gotz, S., Tarazona,
595 S., Dopazo, J., Meyer, T.F., Conesa, A. (2012). Qualimap: evaluating next-generation
596 sequencing alignment data. Bioinformatics. 28 2678-2679.

597 [11] Greub, G., Mege, J.L., Gorvel, J.P., Raoult, D., Meresse, S. (2005). Intracellular
598 trafficking of *Parachlamydia acanthamoebae*. Cell Microbiol. 7 581-589.

599 [12] Greub, G., Mege, J.L., Raoult, D. (2003). *Parachlamydia acanthamoebae* enters and
600 multiplies within human macrophages and induces their apoptosis [corrected]. Infect
601 Immun. 71 5979-5985.

602 [13] Greub, G., Raoult, D. (2002). Crescent bodies of *Parachlamydia acanthamoeba* and
603 its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Appl
604 Environ Microbiol. 68 3076-3084.

605 [14] Horn, M., Wagner, M., Muller, K.D., Schmid, E.N., Fritsche, T.R., Schleifer, K.H.,
606 Michel, R. (2000). *Neochlamydia hartmannellae* gen. nov., sp. nov.
607 (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*.
608 Microbiology. 146 (Pt 5) 1231-1239.

609 [15] Hulin, V., Oger, S., Vorimore, F., Aaziz, R., de Barbeyrac, B., Berruchon, J., Sachse,
610 K., Laroucau, K. (2015). Host preference and zoonotic potential of *Chlamydia psittaci*
611 and *C. gallinacea* in poultry. Pathog Dis. 73 1-11.

612 [16] Hunter, J.D. (2007). Matplotlib: A 2D Graphics Environment. Computing in Science
613 & Engineering. vol. 9 90-95.

614 [17] Isaksson, J., Christerson, L., Blomqvist, M., Wille, M., Alladio, L.A., Sachse, K.,
615 Olsen, B., González-Acuña, D., Herrmann, B. (2015). Chlamydiaceae-like bacterium, but
616 no *Chlamydia psittaci*, in sea birds from Antarctica. *Polar Biology*. 38 1931-1936.

617 [18] Kaleta, E.F., Taday, E.M. (2003). Avian host range of *Chlamydophila* spp. based on
618 isolation, antigen detection and serology. *Avian Pathol.* 32 435-461.

619 [19] Katoh, K., Standley, D.M. (2013). MAFFT multiple sequence alignment software
620 version 7: improvements in performance and usability. *Mol Biol Evol.* 30 772-780.

621 [20] Kolmogorov, M., Yuan, J., Lin, Y., Pevzner, P.A. (2019). Assembly of long, error-
622 prone reads using repeat graphs. *Nat Biotechnol.* 37 540-546.

623 [21] Konstantinidis, K.T., Tiedje, J.M. (2005). Towards a genome-based taxonomy for
624 prokaryotes. *J Bacteriol.* 187 6258-6264.

625 [22] Laroucau, K., Ortega, N., Vorimore, F., Aaziz, R., Mitura, A., Szymanska-
626 Czerwinska, M., Cicerol, M., Salinas, J., Sachse, K., Caro, M.R. (2020). Detection of a
627 novel *Chlamydia* species in captive spur-thighed tortoises (*Testudo graeca*) in
628 southeastern Spain and proposal of *Candidatus Chlamydia testudinis*. *Syst Appl*
629 *Microbiol.* 126071.

630 [23] Laroucau, K., Vorimore, F., Aaziz, R., Solmonson, L., Hsia, R.C., Bavoil, P.M.,
631 Fach, P., Holzer, M., Wuenschmann, A., Sachse, K. (2019). *Chlamydia buteonis*, a new
632 *Chlamydia* species isolated from a red-shouldered hawk. *Syst Appl Microbiol.*

633 [24] Li, H. (2018). Minimap2: pairwise alignment for nucleotide sequences.
634 *Bioinformatics.* 34 3094-3100.

635 [25] Li, H., Durbin, R. (2009). Fast and accurate short read alignment with Burrows-
636 Wheeler transform. *Bioinformatics.* 25 1754-1760.

637 [26] Lienard, J., Croxatto, A., Prod'hom, G., Greub, G. (2011). *Estrella lausannensis*, a
638 new star in the Chlamydiales order. *Microbes Infect.* 13 1232-1241.

639 [27] Luo, C., Rodriguez, R.L., Konstantinidis, K.T. (2014). MyTaxa: an advanced
640 taxonomic classifier for genomic and metagenomic sequences. *Nucleic Acids Res.* 42
641 e73.

642 [28] Martinez, F.V., Feijao, P., Braga, M.D., Stoye, J. (2015). On the family-free DCJ
643 distance and similarity. *Algorithms Mol Biol.* 10 13.

644 [29] Matsumoto, A. (1981). Isolation and electron microscopic observations of
645 intracytoplasmic inclusions containing *Chlamydia psittaci*. *J Bacteriol.* 145 605-612.

646 [30] Merkel, D. (2014). Docker: lightweight Linux containers for consistent development
647 and deployment. *Linux J.* 2014 Article 2.

648 [31] Mitchell, C.M., Mathews, S.A., Theodoropoulos, C., Timms, P. (2009). In vitro
649 characterisation of koala *Chlamydia pneumoniae*: morphology, inclusion development
650 and doubling time. *Vet Microbiol.* 136 91-99.

651 [32] Nans, A., Saibil, H.R., Hayward, R.D. (2014). Pathogen-host reorganization during
652 *Chlamydia* invasion revealed by cryo-electron tomography. *Cell Microbiol.* 16 1457-
653 1472.

654 [33] Onorini, D., Donati, M., Marti, H., Biondi, R., Levi, A., Nufer, L., Prahauer, B.,
655 Rigamonti, S., Vicari, N., Borel, N. (2019). The influence of centrifugation and
656 incubation temperatures on various veterinary and human chlamydial species. *Vet*
657 *Microbiol.* 233 11-20.

658 [34] Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.,
659 Fookes, M., Falush, D., Keane, J.A., Parkhill, J. (2015). Roary: rapid large-scale
660 prokaryote pan genome analysis. *Bioinformatics*. 31 3691-3693.

661 [35] Pantchev, A., Sting, R., Bauerfeind, R., Tyczka, J., Sachse, K. (2009). New real-time
662 PCR tests for species-specific detection of *Chlamydophila psittaci* and *Chlamydophila*
663 *abortus* from tissue samples. *Vet J*. 181 145-150.

664 [36] Pillonel, T., Bertelli, C., Salamin, N., Greub, G. (2015). Taxogenomics of the order
665 Chlamydiales. *Int J Syst Evol Microbiol*. 65 1381-1393.

666 [37] Qin, Q.L., Xie, B.B., Zhang, X.Y., Chen, X.L., Zhou, B.C., Zhou, J., Oren, A.,
667 Zhang, Y.Z. (2014). A proposed genus boundary for the prokaryotes based on genomic
668 insights. *J Bacteriol*. 196 2210-2215.

669 [38] Richter, M., Rossello-Mora, R. (2009). Shifting the genomic gold standard for the
670 prokaryotic species definition. *Proc Natl Acad Sci U S A*. 106 19126-19131.

671 [39] Rodriguez, R.L., Gunturu, S., Harvey, W.T., Rossello-Mora, R., Tiedje, J.M., Cole,
672 J.R., Konstantinidis, K.T. (2018). The Microbial Genomes Atlas (MiGA) webserver:
673 taxonomic and gene diversity analysis of Archaea and Bacteria at the whole genome
674 level. *Nucleic Acids Res*. 46 W282-W288.

675 [40] Rusconi, B., Lienard, J., Aeby, S., Croxatto, A., Bertelli, C., Greub, G. (2013).
676 Crescent and star shapes of members of the Chlamydiales order: impact of fixative
677 methods. *Antonie Van Leeuwenhoek*. 104 521-532.

678 [41] Sachse, K., Laroucau, K., Riege, K., Wehner, S., Dilcher, M., Creasy, H.H.,
679 Weidmann, M., Myers, G., Vorimore, F., Vicari, N., Magnino, S., Liebler-Tenorio, E.,
680 Ruettger, A., Bavoil, P.M., Hufert, F.T., Rossello-Mora, R., Marz, M. (2014). Evidence
681 for the existence of two new members of the family *Chlamydiaceae* and proposal of
682 *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. *Syst Appl Microbiol*. 37
683 79-88.

684 [42] Sachse, K., Laroucau, K., Vanrompay, D. (2015). Avian Chlamydiosis. in: *Current*
685 *Clinical Microbiology Reports*. pp. 10-21.

686 [43] Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics*.
687 30 2068-2069.

688 [44] Spencer, W.N., Johnson, F.W. (1983). Simple transport medium for the isolation of
689 *Chlamydia psittaci* from clinical material. *Vet Rec*. 113 535-536.

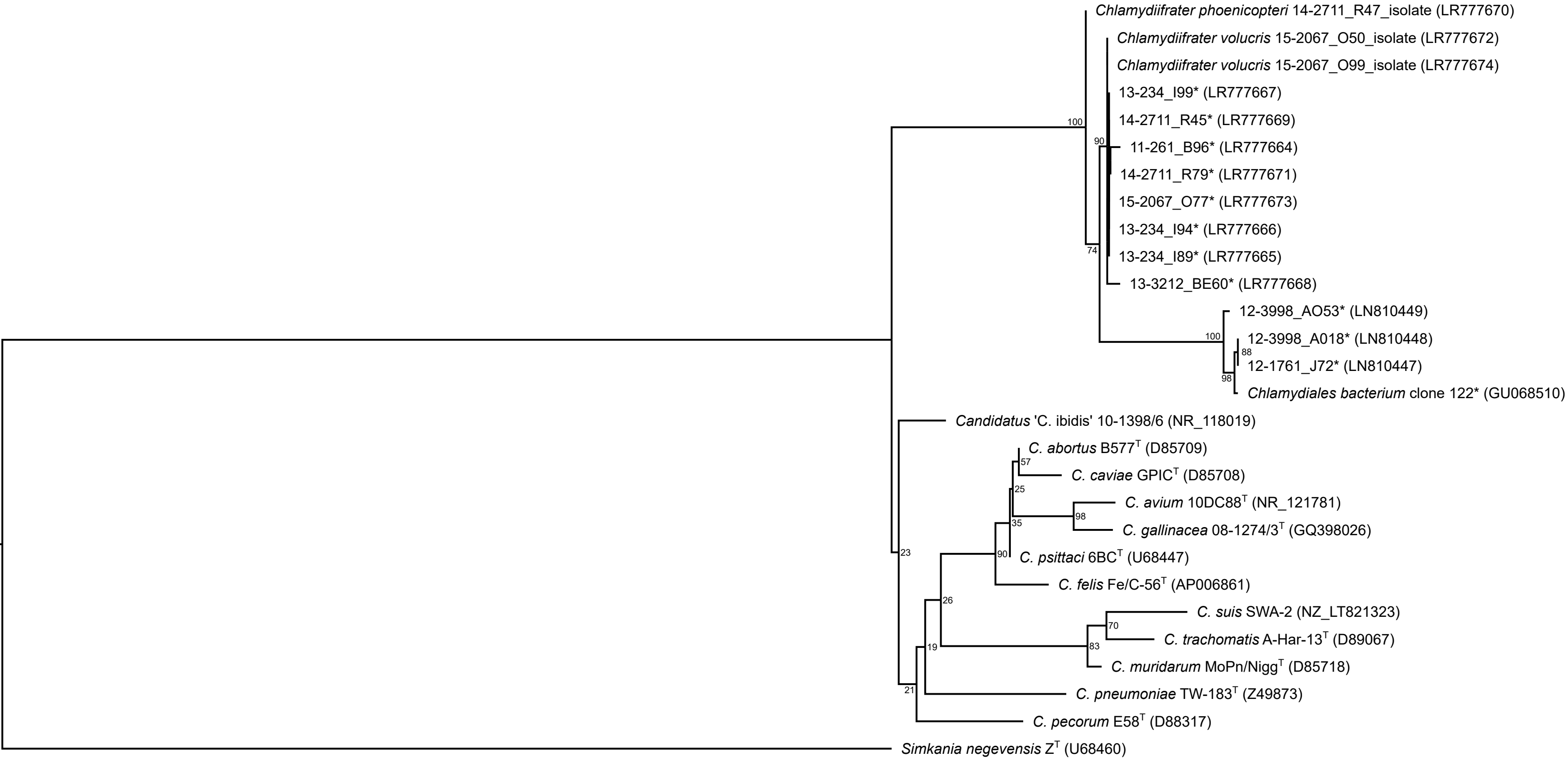
690 [45] Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-
691 analysis of large phylogenies. *Bioinformatics*. 30 1312-1313.

692 [46] Staub, E., Marti, H., Biondi, R., Levi, A., Donati, M., Leonard, C.A., Ley, S.D.,
693 Pillonel, T., Greub, G., Seth-Smith, H.M.B., Borel, N. (2018). Novel *Chlamydia* species
694 isolated from snakes are temperature-sensitive and exhibit decreased susceptibility to
695 azithromycin. *Sci Rep*. 8 5660.

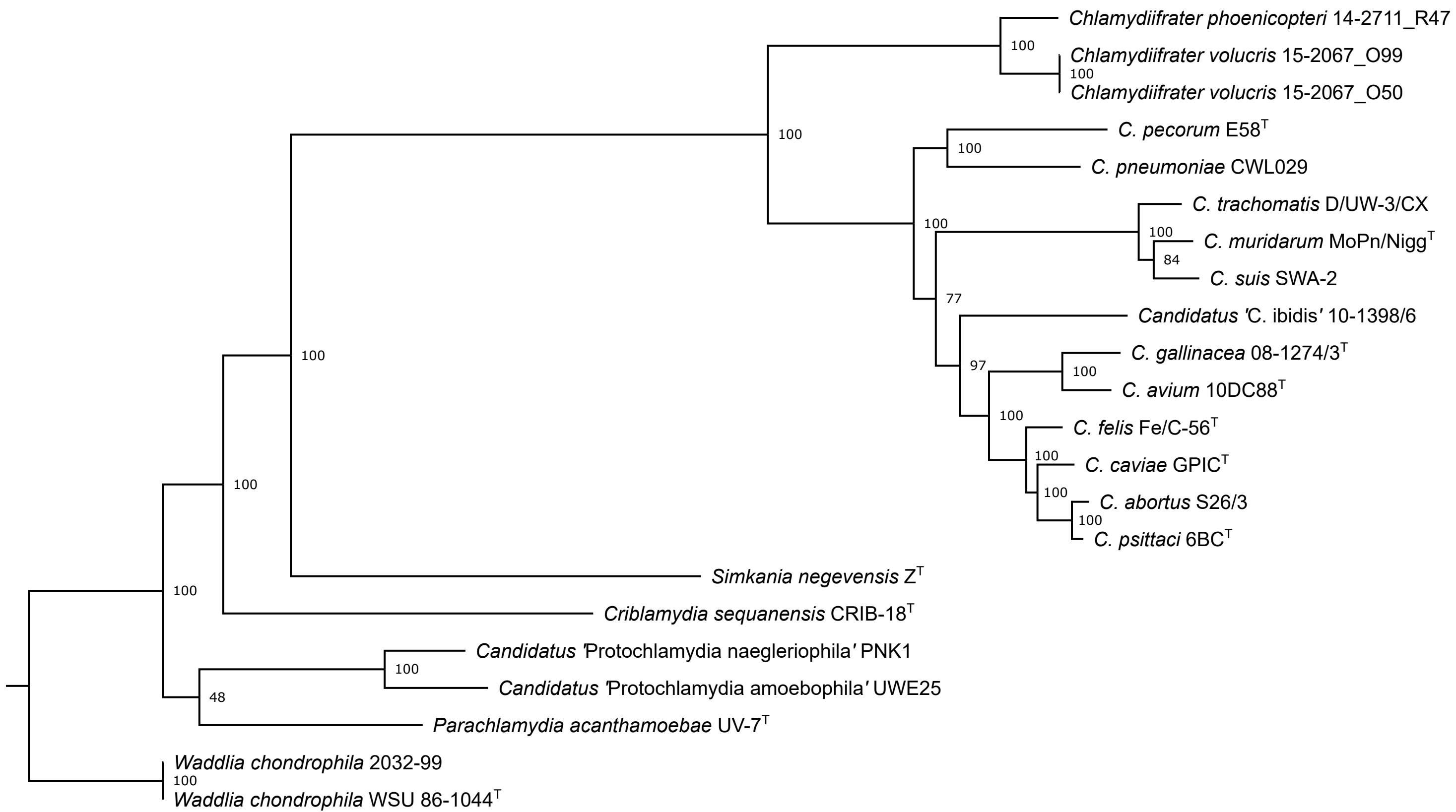
696 [47] Sullivan, M.J., Petty, N.K., Beatson, S.A. (2011). Easyfig: a genome comparison
697 visualizer. *Bioinformatics*. 27 1009-1010.

698 [48] Szymanska-Czerwinska, M., Mitura, A., Niemczuk, K., Zareba, K., Jodelko, A.,
699 Pluta, A., Scharf, S., Vitek, B., Aaziz, R., Vorimore, F., Laroucau, K., Schnee, C. (2017).
700 Dissemination and genetic diversity of chlamydial agents in Polish wildfowl: Isolation
701 and molecular characterisation of avian *Chlamydia abortus* strains. *PLoS One*. 12
702 e0174599.

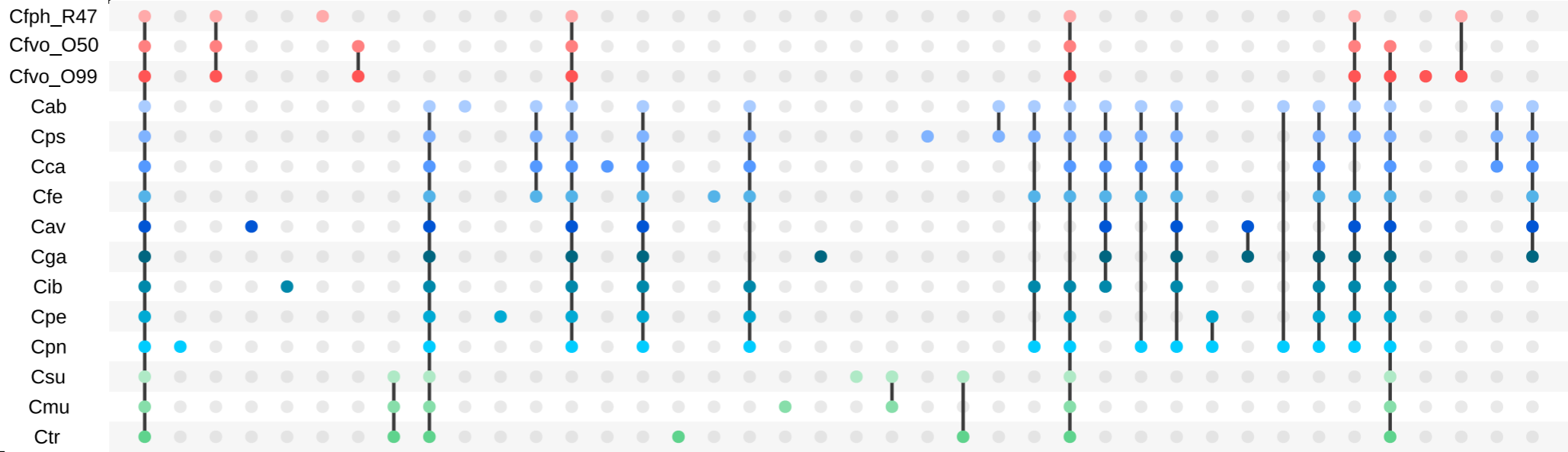
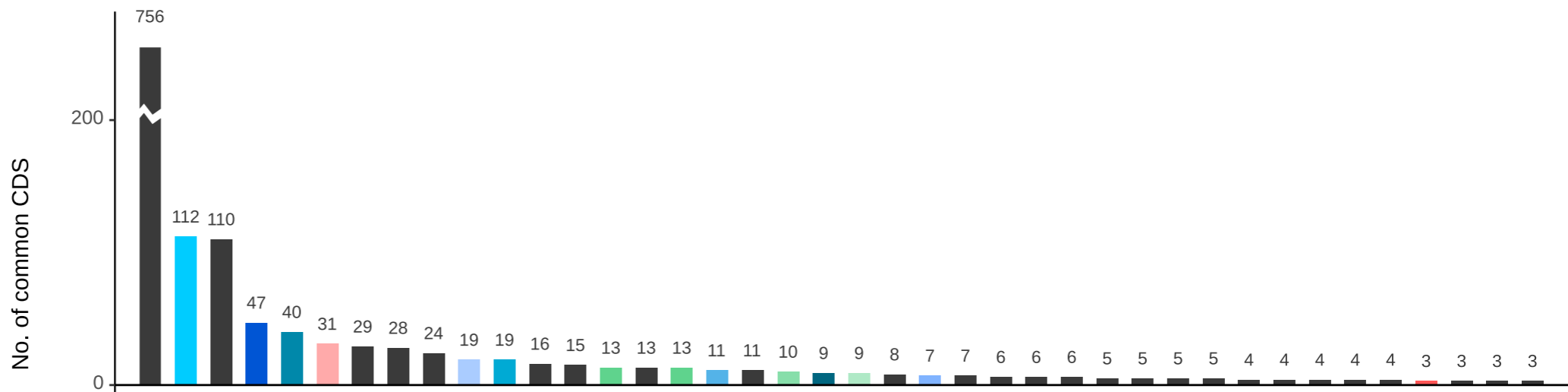
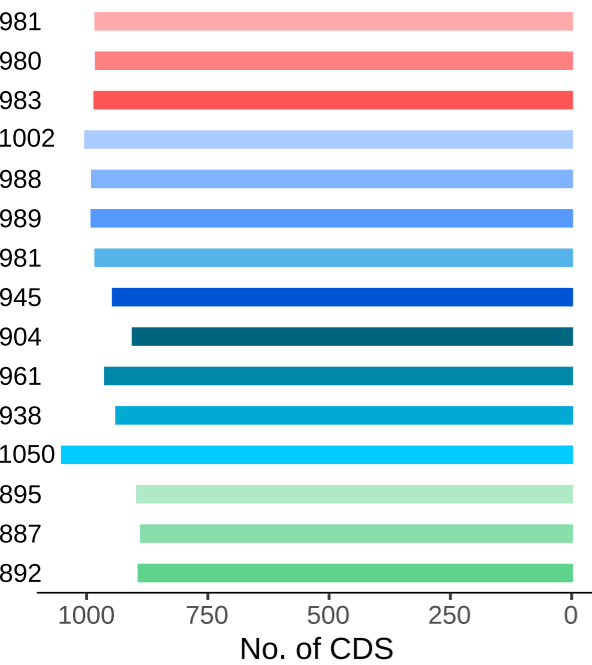
703 [49] Taylor-Brown, A., Bachmann, N.L., Borel, N., Polkinghorne, A. (2016). Culture-
704 independent genomic characterisation of *Candidatus Chlamydia sanzinia*, a novel
705 uncultivated bacterium infecting snakes. *BMC Genomics*. 17 710.
706 [50] Vaser, R., Sovic, I., Nagarajan, N., Sikic, M. (2017). Fast and accurate de novo
707 genome assembly from long uncorrected reads. *Genome Res*. 27 737-746.
708 [51] Vorimore, F., Hsia, R.C., Huot-Creasy, H., Bastian, S., Deruyter, L., Passet, A.,
709 Sachse, K., Bavoil, P., Myers, G., Laroucau, K. (2013). Isolation of a New *Chlamydia*
710 species from the Feral Sacred Ibis (*Threskiornis aethiopicus*): *Chlamydia ibidis*. *PLoS*
711 *One*. 8 e74823.
712 [52] Wick, R.R., Judd, L.M., Gorrie, C.L., Holt, K.E. (2017). Unicycler: Resolving
713 bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol*.
714 13 e1005595.
715 [53] Yarza, P., Yilmaz, P., Pruesse, E., Glockner, F.O., Ludwig, W., Schleifer, K.H.,
716 Whitman, W.B., Euzéby, J., Amann, R., Rossello-Mora, R. (2014). Uniting the
717 classification of cultured and uncultured bacteria and archaea using 16S rRNA gene
718 sequences. *Nat Rev Microbiol*. 12 635-645.
719 [54] Zocevic, A., Vorimore, F., Marhold, C., Horvatek, D., Wang, D., Slavec, B.,
720 Prentza, Z., Stavianis, G., Prukner-Radovcic, E., Dovc, A., Siarkou, V.I., Laroucau, K.
721 (2012). Molecular characterization of atypical *Chlamydia* and evidence of their
722 dissemination in different European and Asian chicken flocks by specific real-time PCR.
723 *Environ Microbiol*. 14 2212-2222.
724 [55] Zocevic, A., Vorimore, F., Vicari, N., Gasparini, J., Jacquin, L., Sachse, K.,
725 Magnino, S., Laroucau, K. (2013). A real-time PCR assay for the detection of atypical
726 strains of *Chlamydiaceae* from pigeons. *PLoS One*. 8 e58741.
727 [56] Zweifel, D., Hoop, R., Sachse, K., Pospischil, A., Borel, N. (2009). Prevalence of
728 *Chlamydia psittaci* in wild birds-potential risk for domestic poultry, pet birds, and
729 public health? *European Journal of Wildlife Research*. 55 575-581.
730
731

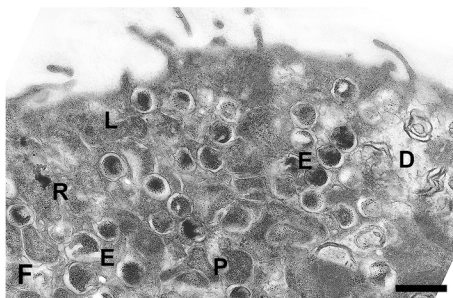
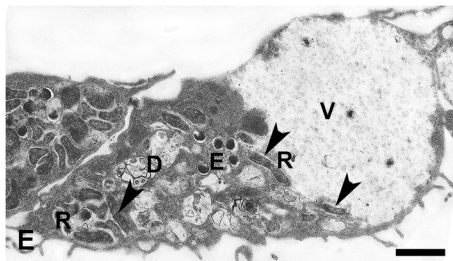
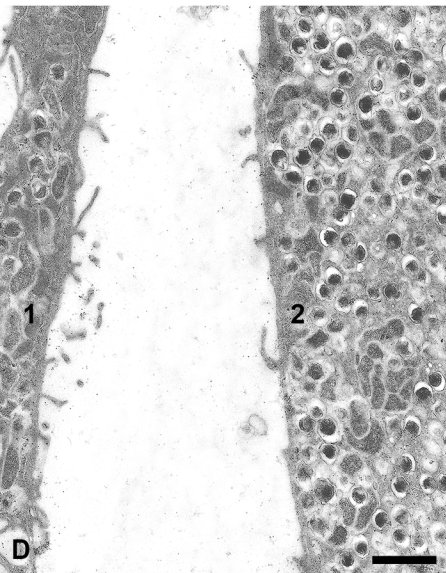
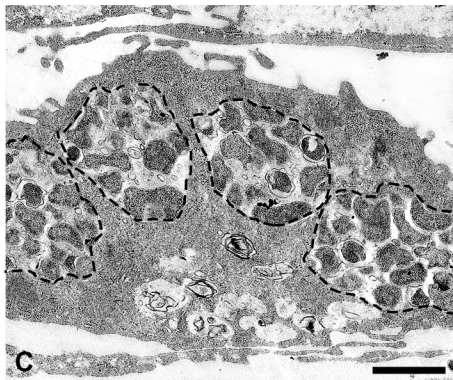
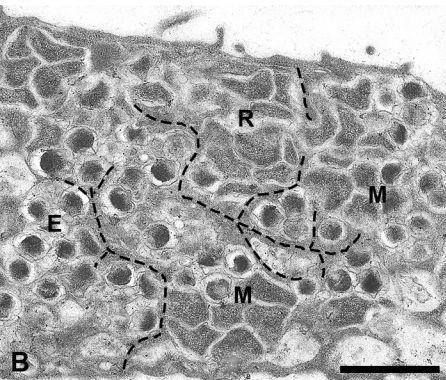
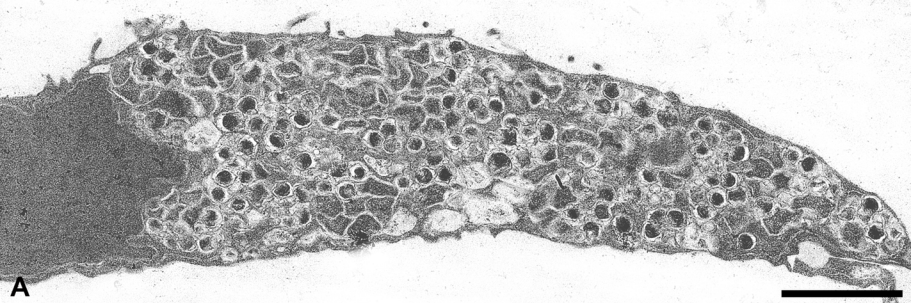


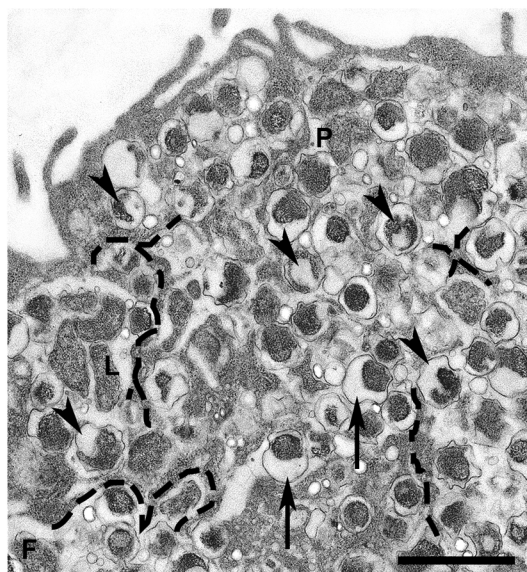
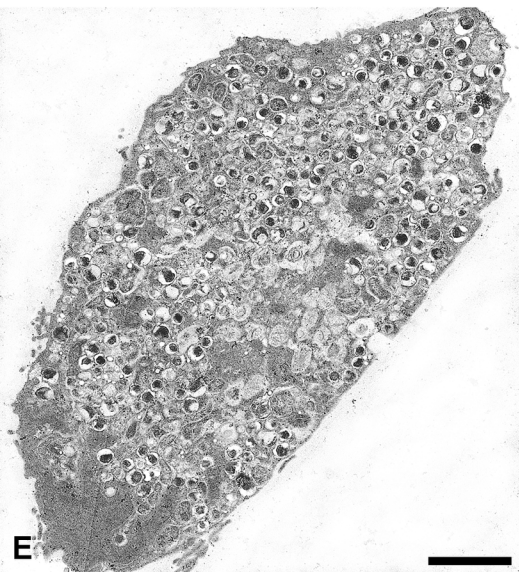
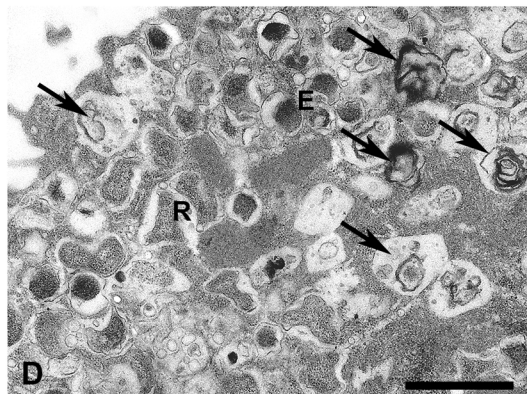
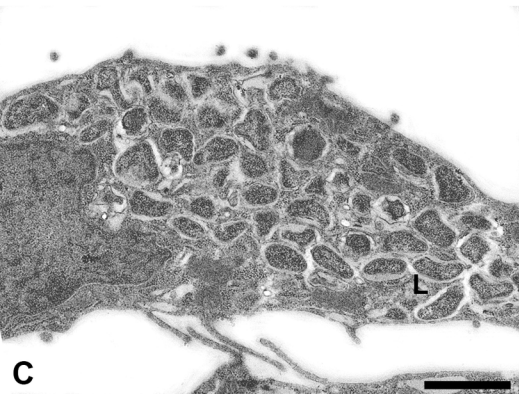
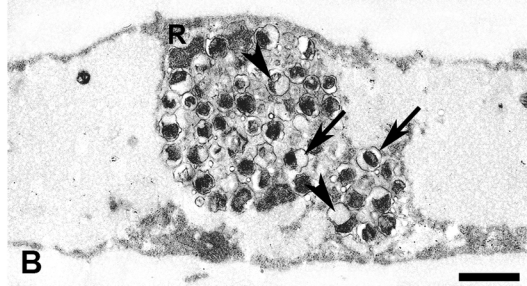
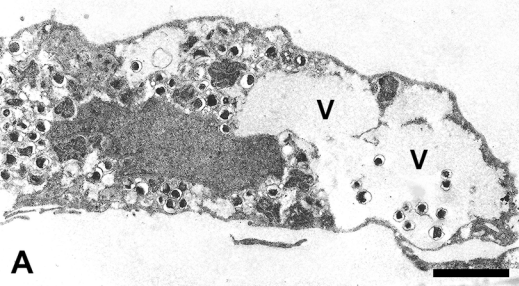
0.04



0.2







Batch ID	Date	Sampling site	Chlamydiaceae PCR			Chlamydiifrater genus-specific PCR			Chlamydiifrater phoenicopteri species-specific PCR			Chlamydiifrater volucris species-specific PCR			Isolation
			+ves/total no.	mean Cq	Cq range	+ves/total no. +ves	mean Cq	Cq range	+ves/total no. +ves	mean Cq	Cq range	+ves/total no. +ves	mean Cq	Cq range	
<u>Preliminary investigation</u>															
11-261	31/01/2011	Zoo - Doué la Fontaine	3/3	31.9	[31.4 - 32.6]	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
<u>Catches</u>															
11-324	04/02/2011	Zoo - Doué la Fontaine	10/15	36.8	[33.6 - 39.6]	8/10	36.7	[33.6 - 39.8]	8/10	34.3	[31 - 37.3]	6/10	35.4	[33.9 - 37.1]	-
13-234	08/08/2012	Etang du Fangassier	48/99	37.0	[31.0 - 39.9]	40/48	37.4	[32.2 - 40.0]	0/48	-	-	44/48	34.0	[29.1 - 36.9]	nd
13-3212	07/08/2013	Etang du Fangassier	19/68	36.8	[30.6 - 40.0]	16/19	36.7	[31.5 - 39.0]	0/19	-	-	17/19	33.7	[27.9 - 37.6]	-
14-1167	21/03/2014	Zoo - Paris	1/37	36.3	-	1/1	35.8	-	0/1	-	-	1/1	33.1	-	-
14-2711	06/08/2014	Salins d'Aigues mortes	17/96	33.6	[26.9 - 39.0]	14/17	35.2	[30.2 - 39.7]	4/17	33.3	[29.2 - 38.1]	11/17	31.3	[26.4 - 35.7]	+ (1 isolate)
15-2067	29/07/2015	Etang du Fangassier	41/141	35.3	[27.6 - 39.9]	36/41	35.2	[28.3 - 39.7]	0/41	-	-	39/41	32.4	[25.0 - 37.7]	+ (2 isolates)
Total			136/456 (29.8%)			115/136 (84.6%)			13/136 (9.6%)			118/136 (86.8%)			
wild birds			125/404 (30.9%)			106/125 (84.8%)			4/125 (3.2%)			111/125 (88.8%)			
captive birds			11/52 (21.1%)			9/11 (81.8%)			9/11 (81.8%)			7/11 (63.6%)			

	<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047	<i>Chlamydiifrater volucris</i> 15-2067_O50	<i>Chlamydiifrater volucris</i> 15-2067_O99	<i>C. avium</i> 10DC88 ^T	<i>C. gallinacea</i> 08-1274/3 ^T	<i>C. psittaci</i> 6BC ^T	<i>C. abortus</i> S26/3	<i>C. caviae</i> GPIC ^T	<i>C. felis</i> Fe/C-56 ^T	<i>C. pecorum</i> E58 ^T	<i>C. pneumoniae</i> TW-183 ^T	<i>C. trachomatis</i> D/UW-3/CX	<i>C. muridarum</i> MoPn/Nigg ^T	<i>C. suis</i> SWA-2	<i>C. ibidis</i> 10-1398/6
<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047		99.32	99.32	94.81	94.80	95.43	95.13	94.69	94.77	95.03	95.09	93.28	93.71	92.92	95.39
<i>Chlamydiifrater volucris</i> 15-2067_O50	99.55		100.00	94.52	94.48	95.28	94.99	94.45	94.81	95.03	94.98	92.69	93.45	92.53	95.60
<i>Chlamydiifrater volucris</i> 15-2067_O99	99.55	100.00		94.52	94.48	95.28	94.99	94.45	94.81	95.03	94.98	92.69	93.45	92.53	95.60
<i>C. avium</i> 10DC88 ^T	94.89	94.47	94.47		98.77	97.19	96.87	96.27	96.48	95.83	96.44	93.23	93.58	93.09	96.18
<i>C. gallinacea</i> 08-1274/3 ^T	94.66	94.66	94.66	98.24		96.69	96.53	95.99	96.17	95.47	96.06	93.08	93.41	92.83	95.75
<i>C. psittaci</i> 6BC ^T	94.85	94.50	94.50	98.16	98.24		99.49	98.39	98.56	96.19	97.26	93.13	94.24	93.42	96.76
<i>C. abortus</i> S26/3	94.06	93.84	93.84	97.81	98.03	99.65		98.04	98.25	96.19	96.99	92.89	93.89	92.86	96.58
<i>C. caviae</i> GPIC ^T	94.57	94.36	94.36	97.83	97.67	98.89	98.88		98.53	95.51	96.85	92.83	93.81	93.37	96.30
<i>C. felis</i> Fe/C-56 ^T	94.21	93.76	93.76	97.09	97.09	98.53	98.17	98.03		95.69	96.95	92.74	93.74	92.95	96.62
<i>C. pecorum</i> E58 ^T	94.35	94.20	94.20	96.28	96.07	96.43	96.00	95.99	95.92		96.15	92.31	93.13	92.53	95.83
<i>C. pneumoniae</i> TW-183 ^T	93.93	93.79	93.79	94.59	94.48	95.97	95.39	95.03	94.79	95.32		93.09	94.11	93.13	96.26
<i>C. trachomatis</i> D/UW-3/CX	93.91	93.77	93.77	95.29	94.94	95.67	95.33	95.33	94.67	95.04	94.80		97.95	98.17	93.39
<i>C. muridarum</i> MoPn/Nigg ^T	93.86	93.99	93.99	95.51	95.11	96.02	95.63	95.75	95.34	95.41	94.38	98.57		97.90	94.15
<i>C. suis</i> SWA-2	93.57	93.29	93.29	95.01	94.27	95.46	94.93	94.97	94.87	94.27	93.35	97.63	97.97		93.41
<i>C. ibidis</i> 10-1398/6	95.64	95.57	95.57	96.53	96.73	97.22	96.81	96.68	96.23	96.58	96.25	95.31	95.65	95.00	

	<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047	<i>Chlamydiifrater volucris</i> 15-2067_O50	<i>Chlamydiifrater volucris</i> 15-2067_O99	<i>C. avium</i> 10DC88 ^T	<i>C. gallinacea</i> 08-1274/3 ^T	<i>C. psittaci</i> 6BC ^T	<i>C. abortus</i> S26/3	<i>C. caviae</i> GPIC ^T	<i>C. felis</i> Fe/C-56 ^T	<i>C. pecorum</i> E58 ^T	<i>C. pneumoniae</i> TW-183 ^T	<i>C. trachomatis</i> D/UW-3/CX	<i>C. muridarum</i> MoPn/Nigg ^T	<i>C. suis</i> SWA-2	<i>C. ibidis</i> 10-1398/6
Genome size [bp]	1,187,976	1,182,155	1,195,261	1,041,170	1,059,583	1,171,667	1,144,377	1,173,390	1,166,239	1,106,197	1,225,935	1,042,519	1,072,950	1,075,789	1,146,066
G+C [mol-%]	40.30	40.42	40.41	36.92	37.94	39.06	39.87	39.22	39.38	41.08	40.58	41.31	40.34	42.08	38.32
CDS	981	980	983	945	904	988	1002	989	981	938	1050	892	887	895	961
5S, 16S, 23S rRNA	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	1, 1, 1	2, 2, 2	2, 2, 2	2, 2, 2	1, 1, 1
tRNA	39	38	38	39	39	39	38	38	38	38	38	37	37	37	38
tmRNA	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
plasmid	1	1	1	1	1	1	0	1	1	0	0	0	1	1	1

	<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047	<i>Chlamydiifrater volucris</i> 15-2067_O50	<i>Chlamydiifrater volucris</i> 15-2067_O99	<i>C. avium</i> 10DC88 ^T	<i>C. gallinacea</i> 08-1274/3 ^T	<i>C. psittaci</i> 6BC ^T	<i>C. abortus</i> S26/3	<i>C. caviae</i> GPIC ^T	<i>C. felis</i> Fe/C-56 ^T	<i>C. pecorum</i> E58 ^T	<i>C. pneumoniae</i> TW-183 ^T	<i>C. trachomatis</i> D/UW-3/CX	<i>C. muridarum</i> MoPn/Nigg ^T	<i>C. suis</i> SWA-2	<i>C. ibidis</i> 10-1398/6
<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047		0.9460	0.9452	0.7126	0.7112	0.7520	0.7348	0.7458	0.7523	0.6957	0.6554	0.7956	0.8097	0.8370	0.7590
<i>Chlamydiifrater volucris</i> 15-2067_O50	75.53		0.9997	0.7540	0.7467	0.7632	0.7508	0.7480	0.7588	0.6831	0.6294	0.7662	0.8006	0.7932	0.8379
<i>Chlamydiifrater volucris</i> 15-2067_O99	75.58	99.46		0.7554	0.7473	0.7633	0.7509	0.7485	0.7592	0.6835	0.6277	0.7661	0.8005	0.7928	0.8389
<i>C. avium</i> 10DC88 ^T	66.03	65.89	65.80		0.9864	0.9235	0.8983	0.9007	0.9276	0.8114	0.8093	0.7645	0.8060	0.7785	0.9329
<i>C. gallinacea</i> 08-1274/3 ^T	65.86	65.72	65.77	81.09		0.9413	0.9233	0.9114	0.9475	0.8332	0.8325	0.7869	0.8217	0.8001	0.9258
<i>C. psittaci</i> 6BC ^T	65.83	65.83	65.74	73.86	73.34		0.9860	0.9698	0.9665	0.8831	0.8961	0.8410	0.8546	0.8332	0.8865
<i>C. abortus</i> S26/3	65.87	65.79	65.78	73.57	73.33	92.58		0.9565	0.9567	0.8832	0.9039	0.8334	0.8357	0.8213	0.8565
<i>C. caviae</i> GPIC ^T	65.95	66.05	65.70	74.01	73.83	81.10	80.75		0.9510	0.8921	0.9114	0.8334	0.8440	0.8198	0.8488
<i>C. felis</i> Fe/C-56 ^T	65.88	65.71	65.63	73.70	73.50	80.75	80.44	81.96		0.8739	0.8769	0.8292	0.8433	0.8365	0.8836
<i>C. pecorum</i> E58 ^T	65.69	65.70	65.75	68.39	68.19	68.84	68.87	69.08	68.83		0.9022	0.7881	0.7761	0.7761	0.7294
<i>C. pneumoniae</i> TW-183 ^T	65.65	66.07	65.88	68.97	68.87	69.74	69.66	69.85	69.76	69.32		0.7864	0.7968	0.7855	0.7106
<i>C. trachomatis</i> D/UW-3/CX	65.99	66.00	65.76	68.12	67.97	68.38	68.23	68.62	68.20	67.13	67.52		0.9728	0.9714	0.7690
<i>C. muridarum</i> MoPn/Nigg ^T	66.20	65.98	66.01	68.30	68.06	68.39	68.17	68.72	68.43	67.13	67.57	80.23		0.9770	0.8077
<i>C. suis</i> SWA-2	65.74	66.12	65.78	67.78	67.77	68.12	67.97	68.02	68.13	66.76	67.29	79.14	81.41		0.7728
<i>C. ibidis</i> 10-1398/6	65.84	66.20	66.11	69.91	69.87	70.72	70.57	70.81	70.66	67.88	68.52	68.01	68.05	67.78	

	<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047	<i>Chlamydiifrater volucris</i> 15-2067_O50	<i>Chlamydiifrater volucris</i> 15-2067_O99	<i>C. avium</i> 10DC88 ^T	<i>C. gallinacea</i> 08-1274/3 ^T	<i>C. psittaci</i> 6BC ^T	<i>C. abortus</i> S26/3	<i>C. caviae</i> GPIC ^T	<i>C. felis</i> Fe/C-56 ^T	<i>C. pecorum</i> E58 ^T	<i>C. pneumoniae</i> TW-183 ^T	<i>C. trachomatis</i> D/UW-3/CX	<i>C. muridarum</i> MoPn/Nigg ^T	<i>C. suis</i> SWA-2	<i>C. ibidis</i> 10-1398/6
<i>Chlamydiifrater phoenicopteri</i> 14-2711_R047		95.41	95.26	70.82	71.19	71.25	70.70	70.46	70.23	71.29	67.65	69.41	69.16	69.03	70.70
<i>Chlamydiifrater volucris</i> 15-2067_O50	80.45		99.85	70.65	70.91	70.53	69.17	69.68	69.76	70.70	66.65	69.34	68.88	68.64	70.43
<i>Chlamydiifrater volucris</i> 15-2067_O99	80.51	99.58		70.54	70.80	70.27	69.07	69.62	69.65	70.59	66.55	69.33	68.77	68.53	70.32
<i>C. avium</i> 10DC88 ^T	55.96	56.03	55.97		96.65	90.22	89.32	89.25	89.56	86.09	81.20	83.78	83.19	82.99	85.83
<i>C. gallinacea</i> 08-1274/3 ^T	55.89	55.98	56.02	83.71		90.43	88.98	89.96	90.50	87.46	82.65	84.19	83.70	83.49	87.13
<i>C. psittaci</i> 6BC ^T	56.29	56.45	56.54	74.42	74.06		98.64	96.81	96.44	86.09	82.83	82.50	82.56	82.42	87.74
<i>C. abortus</i> S26/3	56.11	56.32	56.35	74.17	73.87	93.68		95.43	94.81	85.15	82.31	81.42	81.00	81.13	86.55
<i>C. caviae</i> GPIC ^T	56.23	56.19	56.20	74.60	74.10	84.71	84.38		96.19	86.30	82.93	82.88	82.89	83.07	86.67
<i>C. felis</i> Fe/C-56 ^T	56.39	56.45	56.50	74.45	74.23	84.17	83.98	85.35		87.34	83.36	82.92	83.03	82.62	86.77
<i>C. pecorum</i> E58 ^T	56.04	56.09	56.02	65.30	65.38	66.73	66.80	66.94	67.02		85.01	82.40	82.52	82.38	84.47
<i>C. pneumoniae</i> TW-183 ^T	56.16	56.16	56.13	66.22	66.19	67.92	67.64	68.38	68.00	67.63		77.34	77.39	76.66	80.76
<i>C. trachomatis</i> D/UW-3/CX	55.41	55.50	55.46	63.72	63.47	64.95	64.78	64.91	65.00	62.05	62.42		96.40	97.09	81.81
<i>C. muridarum</i> MoPn/Nigg ^T	55.30	55.37	55.37	63.82	63.71	65.05	64.72	64.99	65.10	62.16	62.62	85.35		96.91	82.14
<i>C. suis</i> SWA-2	55.31	55.44	55.49	63.69	63.71	64.99	64.67	64.90	64.72	62.13	62.40	84.68	86.06		81.36
<i>C. ibidis</i> 10-1398/6	56.14	56.14	56.11	67.90	67.91	69.97	69.74	70.13	70.07	65.04	65.86	63.67	63.70	63.60	

Table 7 Characteristic traits and features of *Chlamydiifrater* gen.nov. in comparison to the genus *Chlamydia*

	<i>Chlamydiifrater</i>	<i>Chlamydia</i>
No. species	2	11 spp. validly published + 3 spp. + 4 <i>Candidatus</i>
Natural hosts	flamingo (<i>Phoenicopterus</i> spp.)	mammals (incl. humans), birds, amphibians, reptiles
Pathogenicity	unknown (no evidence of pathogenicity to date)	severe, mild, non-pathogenic
Lifestyle	obligate intracellular	obligate intracellular
Morphology	numerous confluent inclusions per host cell, each containing only a few chlamydial bodies	one or a few inclusions per host cell, each harboring up to 10 ² chlamydial bodies
	electron-dense elementary bodies of oval or circular shape and pleomorphic electron-lucid reticulate bodies of elongated shape	electron-dense elementary bodies and electron-lucid reticulate bodies, both of oval or circular shape
Genome size (Mbp)	1.18 to 1.19	1.04 (<i>C. trachomatis</i>) to 1.23 (<i>C. pneumoniae</i>)
16S rRNA sequence identity (%)	93.29-95.64	[reference 100]
Average nucleotide identity (ANIb)	65.70-66.20	[reference 100]
Average amino acid identity (AAI)	55.30-56.54	[reference 100]
POCP (%)	66.55-71.29	[reference 100]
<i>ompA</i> locus	one <i>ompA</i> locus plus additional truncated copies	one <i>ompA</i> locus

Genus name	<i>Chlamydiifrater</i>		
Species name	-	<i>Chlamydiifrater phoenicopteri</i>	<i>Chlamydiifrater volucris</i>
Taxon status	gen. nov.	sp. nov.	sp. nov.
Etymology	Chla.my.di.i.fra'ter. N.L. fem. n. <i>Chlamydia</i> a bacterial genus; L. masc. n. <i>frater</i> brother; N.L. masc. n. <i>Chlamydiifrater</i> brother of <i>Chlamydia</i>	phoe.ni.co.pt'e'ri. N.L. gen. n. phoenicopteri of Phoenicopterus	vo.lu'cris. L. gen. n. volucris of a bird
Type species of the genus	<i>Chlamydiifrater phoenicopteri</i>	-	-
Description of the new taxon and diagnostic traits	<p>All members of the genus are obligate intracellular bacteria with a Gram-negative type cell wall. They follow a biphasic developmental cycle of generally longer duration compared to members of the related genus <i>Chlamydia</i>. Along this cycle, inclusions harbor electron-dense, infective elementary bodies, which transform into electron-lucent, metabolically active and replicating reticulate bodies. Intermediate bodies are scarce or absent, at least in the early and middle phases of the cycle. <i>Chlamydiifrater</i> spp. possess a circular genome sized in the order of 1.2 Mbp. Members of this genus are distinguished from <i>Chlamydia</i> spp. and other taxa of the family <i>Chlamydiaceae</i> by their ribosomal RNA sequences (less than 96% 16S and 23S rRNA sequence identities) and the following genomic parameters: ANI (< 67%), Tetra (< 0.84), AAI (< 57%), and POCP (< 72%).</p> <p>The only currently known host of <i>Chlamydiifrater phoenicopteri</i> is the flamingo (<i>Phoenicopterus roseus</i>). The bacterium can be recovered from the contents of the cloaca. Natural routes of transmission have yet to be investigated, but, analogous to most other chlamydiae, airborne transmission through aerosolized dry feces and dust particles seems likely. From the observations made so far, <i>Chlamydiifrater phoenicopteri</i> seems to be a commensal agent. The potential for a facultative pathogenic role and zoonotic infection of humans is unknown. <i>Chlamydiifrater phoenicopteri</i> strains can be grown in cell culture or embryonated chicken eggs. Their growth is characterized by a biphasic developmental cycle, which is encountered in the other chlamydial organisms as well. Elementary bodies and pleomorphic reticulate bodies are distributed in numerous confluent inclusions throughout the cells. Members of the species can be specifically identified using real-time PCR targeting the rpoN (RNA polymerase sigma-54 factor) gene locus. In addition, strains of the species can be identified using 16S rRNA gene sequence analysis.</p> <p>All strains of <i>Chlamydiifrater volucris</i> isolated so far originated from the flamingo (<i>Phoenicopterus roseus</i>). The bacterium can be recovered from the contents of the cloaca. Natural transmission routes have yet to be investigated, but, analogous to most other chlamydiae, airborne transmission through aerosolized dry feces and dust particles seems possible. It appears likely that <i>Chlamydiifrater volucris</i> is a commensal agent in wild birds. The potential for zoonotic infection of humans is unknown. <i>Chlamydiifrater volucris</i> strains can be grown in cell culture or embryonated chicken eggs. Their growth is characterized by a biphasic developmental cycle, which is encountered in <i>Chlamydia</i> spp. and other members of the order <i>Chlamydiales</i>. Elementary bodies and pleomorphic reticulate bodies are distributed in numerous confluent inclusions throughout the cells. Strains belonging to this the species can be specifically identified using real-time PCR targeting the rpoN (RNA polymerase sigma-54 factor) gene locus. In addition, they can be identified by their 16S rRNA gene sequences.</p>		
Country of origin	France	France	France
Region of origin	Camargue	Camargue	Camargue
Date of isolation (dd/mm/yyyy)	09/09/2014	09/09/2014	
Source of isolation	Salin d'Aigues-Mortes	Salin d'Aigues-Mortes	Etang du Fangassier
Sampling date (dd/mm/yyyy)	06/08/2014	06/08/2014	29/07/2015
Latitude (xx°xx'xx"N/S)	43°33'28.9"N	43°33'28.9"N	43°25'33"N
Longitude (xx°xx'xx"E/W)	4°10'56.6"E	4°10'56.6"E	4°37'27"E
Altitude (meters above sea level)	2	2	2
16S rRNA gene accession nr.	LR777670	LR777670	LR777672
Genome accession number	ERZ1300893	ERZ1300893	ERZ1300894
[RefSeq; EMBL; ...]			
Genome status	complete	complete	complete
Genome size	1.19	1.19	1.18
GC mol%	40.3	40.3	40.4
Number of strains in study	1	1	2
Source of isolation of non-type strains	-	-	Etang du Fangassier
Information related to the Nagoya Protocol	-	-	-
Designation of the Type Strain	14-2711_R47 ^T	14-2711_R47 ^T	15-2067_O50 ^T
Strain Collection Numbers	DSMZ: DSM 110503, CSUR: Q1352	DSMZ: DSM 110503, CSUR: Q1352	DSMZ: DSM 110504, CSUR: Q1351