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| **Abstract:** | During a study on the prevalence and diversity of campylobacteria in wild birds faecal samples in the city of Valdivia (South of Chile) 17 Gram-negative, curved rod isolates, were initially identified as Campylobacter lari by PCR-RFLP. Further identification by 16S rRNA sequence analysis revealed that they formed a distinct group in the genus Campylobacter. This unique position was confirmed by rpoB, atpA and cpn60 gene sequence analysis. The average nucleotide identity between the representative strain WBE38T and the type strain of the closest taxon Campylobacter lari subsp. concheus (LMG 11760) was 90.8%. The oxidase and urease activity of the novel isolates enabled them to be phenotypically differentiated from recognized Campylobacter species. Therefore, on the basis of phenotypic, genetic and genomic characterizations, this study clearly shows that these strains represent a novel species within the genus Campylobacter, for which the name Campylobacter ornithocola sp. nov. is proposed, with the type strain WBE38T (=CECT 9147T =LMG 29815T). |

# *Campylobacter ornithocola* sp. nov., a new member of the *Campylobacter lari* group isolated from wild bird faecal samples

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *rpoB*, *atpA*, *cpn60* genes and the draft genome of the strain WBE38$^T$are KX467974, KX467989, KX467979, KX467985 and LXSU00000000, respectively.

## ABSTRACT

During a study on the prevalence and diversity of campylobacteria in wild birds faecal samples in the city of Valdivia (South of Chile) 17 Gram-negative, curved rod isolates, were initially identified as *Campylobacter lari* by PCR-RFLP. Further identification by 16S rRNA sequence analysis revealed that they formed a distinct group in the genus *Campylobacter*. This unique position was confirmed by *rpoB*, *atpA* and *cpn60* gene sequence analysis. The average nucleotide identity between the representative strain WBE38$^T$ and the type strain of the closest taxon *Campylobacter lari* subsp. *concheus* (LMG 11760) was 90.8%. The oxidase and urease activity of the novel isolates enabled them to be phenotypically differentiated from recognized *Campylobacter* species. Therefore, on the basis of phenotypic, genetic and genomic characterizations, this study clearly shows that these strains represent a novel species within the genus *Campylobacter*, for which the name *Campylobacter ornithocola* sp. nov. is proposed, with the type strain WBE38$^T$ (=CECT 9147$^T$ =LMG 29815$^T$).

The genus *Campylobacter* was proposed by Sebald & Veron (1963), to accommodate the species *Vibrio fetus* (now *Campylobacter fetus*, the type species of the genus). Since then the number of *Campylobacter* species has greatly increased and, at the time of writing (September 2016), this genus encompassed 28 species and 9 subspecies with valid published names (LPSN, 2016; Piccirillo *et al*., 2016; Van et al., 2016). The *Campylobacter* infection is the leading cause of foodborne bacterial gastroenteritis in the developed world, where infection is principally the result of consumption of contaminated meat and poultry. The most common symptoms of campylobacteriosis include severe diarrhoea, with a proportion of patients developing chronic sequelae such as Guillain-Barré syndrome, reactive arthritis and irritable bowel syndrome (Man, 2011). *Campylobacter jejuni* and *Campylobacter coli* are by far the most isolated and studied species, while the epidemiology and the clinical role of the other less common species in human and animal diseases is far less understood (Man, 2011; Iraola et al., 2014).

In the present study, 17 new *Campylobacter* isolates recovered from wild bird faecal samples were subjected to a polyphasic approach including molecular identification by PCR-RFLP, genotyping by ERIC-PCR, phylogenetic analysis using the 16S rRNA, *rpoB*, *atpA* and *cpn60* genes and phenotypic characterization, in order to determine their taxonomic position. Additionally, the whole genome of WBE38$^T$ was sequenced, since it was selected as the type strain. Based on our results, we propose and describe these strains as a novel species within the genus *Campylobacter*.

Over the period between January 2013 and October 2015, a study was conducted to evaluate the prevalence and diversity of campylobacteria in wild bird faecal samples in the city of Valdivia, South of Chile, where a collection of *Campylobacter* isolates was obtained

(L. Collado, paper in preparation). The samples corresponded to bird's faecal droppings excreted in public urban parks. Although the specific type of bird corresponding to each specimen remains unidentified, at the time of sampling black-faced ibis (*Theristicus melanopis*), southern lapwing (*Vanellus chilensis*) and chimango caracara (*Milvago chimango*) were the main species observed at these sites. Faecal samples were collected using sterile cotton-tipped swabs that were immediately placed into 8 ml Bolton broth (Oxoid). Incubation was performed at 37 °C for 48 h under micro-aerobic conditions (CampyGen, Oxoid). After enrichment, an aliquot (400 µl) was transferred onto the surface of a 0.45 µm membrane filter (Millipore) which was placed on a Petri dish containing Blood Agar Base (Merck) supplemented with 5% defibrinated sheep blood (Quad Five) and it was allowed to filter passively under room conditions for 30 min. After filtration, the filters were removed with sterile forceps and discarded. The inoculated plates were incubated under the aforementioned conditions (Collado *et al*., 2014). The 17 novel isolates described here were initially identified as *Campylobacter lari* by the PCR-RFLP described in Marshall *et al*. (1999) showing the C1 and C1b restriction patterns with the endonucleases *Dde*I and *Bsr*I, respectively (data not shown). However, recently this species was divided into two subspecies (*C. lari* subsp. *lari* and *C. lari* subsp. *concheus*) (Debuyne *et al*., 2009). Additionally, several *C. lari*-like new species were described, i.e., *C. insulaenigrae* (Foster *et al*., 2004), *C. peloridis* (Debuyne *et al*., 2009), *C. subantarticus* (Debuyne *et al*., 2010a) and *C. volucris* (Debuyne *et al*., 2010b), that collectively have been referred to as *Campylobacter lari* group (Miller *et al*., 2014b). Since no available PCR method could differentiate these highly related taxa, all isolates we identified as *C. lari* by PCR-RFLP were further analyzed by a polyphasic taxonomic approach.

The 17 isolates were first analysed by means of the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) technique using the Houf *et al*. (2002) protocol, in order to avoid further analysis of strains with the same genotype. The electrophoretic patterns were analysed using the Jaccard index and a dendrogram was constructed by using the unweighted pair group linkage analysis (UPGMA) method with the Phoretix 1d Pro software (Totallab). As shown in Fig. 1, all the isolates belonged to different ERIC-PCR types and, consequently, were considered different strains. A nearly complete 16S rRNA gene sequence (1350 bp) of five selected strains (WBE38$^T$, WBE186, WBE206, WBE215 and WBE241), were amplified as described by Vandamme *et al*. (2006). Additionally, the *rpoB*, *atpA* and *cpn60* genes were also evaluated with the protocols described by Korczak *et al*. (2006), Miller *et al*. (2014a) and Hill *et al*. (2006), respectively. Due to the high number of strains, only the analysis of *rpoB* gene sequences included all 17 strains. Both DNA strands of the PCR products were directly sequenced with an ABI 3730 XL automatic DNA sequencer (ABI) by a commercial sequencing facility (Macrogen, Seoul, Korea). Alignment of the sequences was performed with the CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed with the MEGA 6 software (Tamura *et al.*, 2011), by using the neighbour-joining method (Saitou & Nei, 1987) with Kimura's two-parameter substitution model (Kimura, 1980) and the stability of the groupings, estimated by bootstrap analysis (500 replications). Similarity values between the 16S rRNA gene sequence of strain WBE38$^T$ and the type strains of all members of the genus *Campylobacter* with valid published names were calculated with the EzTaxon-e server (Kim *et al.*, 2012) obtaining a similarity range of 99.5- 90.7%. The 16S rDNA phylogenetic tree (Fig. 2) clearly indicated that the five strains represented a single species within the genus *Campylobacter*, most closely related to *C. subantarticus* (99.5% similarity) and *C. lari* subsp. *concheus* (99.4% similarity). The taxonomic position of the new strains within

the *Campylobacter lari* group and the demonstration that they represent a novel lineage were confirmed by the *rpoB*, *atpA* and *cpn60* phylogenetic trees (Supplementary Fig. 1, 2 and 3, respectively).

For the whole genome sequencing of strain WBE38$^T$, genomic libraries were prepared with the Nextera® XT DNA Sample Perparation Kit (Illumina, Inc.) and then sequenced using a MiSeq Illumina platform which produced 5,250,728 pair-end reads (2x150 cycles). After initial quality check reads were assembled with SPAdes (Bankevich *et al*., 2012) and annotated with Prokka (Seemann, 2014), producing 160 contigs that were deposited in the GenBank under the accession number LXSU00000000. The version described in this paper is version LXSU01000000. The average nucleotide identity (ANI) was used as an alternative to DNA-DNA hybridization (DDH) (Konstantinidis & Tiedje, 2005). The ANIb (based on BLAST) values of WBE38$^T$ compared to the sequenced type strains of all other species of the *C. lari* group were calculated using JSpecies v1.2.1 (Richter & Rosselló-Mora, 2009) and were below the 95% species cut-off (Table 1) (Konstantinidis & Tiedje, 2005). The genomic G+C content of WBE38$^T$ was calculated using in-house R scripts and resulted in 29.5%, which is within the range reported for the genus *Campylobacter* (29-47%) (Debruyne *et al*., 2008).

For the physiological and biochemical characterizations of all the new strains, phenotypic testing was performed as described previously (On & Holmes, 1991a, b, 1992; Ursing *et al*., 1995). Growth of strains was determined on nutrient broth no. 2 (Oxoid) supplemented with 5% defibrinated sheep blood (Quad Five) and 2% agar (Merck). Micro-aerobic growth (using CampyGen, Oxoid) was evaluated at 25°C, 37°C and 42°C for 48 to 72h. Aerobic and anaerobic growths (using AnaeroGen, Oxoid) were evaluated at 37°C for 72h.

Catalase activity was evaluated by adding a 3% $H_2O_2$ solution and observing the reaction within 5s. Oxidase activity was determined with Bactident Oxidase strips (Merck). Indoxyl acetate hydrolysis was determined as described by Mills & Gherna (1987). In addition to this, a set of additional phenotypic tests (reduction of nitrates, esterase activity, hydrolysis of hippurate, γ-glutamyl transferase activity, reduction of triphenyl-tetrazolium chloride (TTC), alkaline phosphatase activity, production of $H_2S$, assimilation of glucose and pyrrolidonyl-, L-arginine- and L-aspartate- arylamidase activities) were evaluated by using the API Campy identification system (bioMérieux) according to the manufacturer's instructions. All tests were performed at least twice with *C. jejuni* (DSM 4688$^T$), *C. coli* (DSM 4689$^T$), *C. lari* (DSM 11375$^T$), *C. subantarticus* (LMG 24377$^T$), *C. insulaenigrae* (LMG 22716), *C. volucris* (LMG 24380$^T$) and *Escherichia coli* (ATCC 25922) used as controls. The novel isolates were biochemically different from most of the recognized species of the genus *Campylobacter* because oxidase activity was not detected. This test is positive in all species except *C. gracilis* and sporadic isolates of *C. concisus* and *C. showae* (Vandamme *et al*., 2005). However, the new isolates could be differentiated from oxidase negative campylobacters by the urease test. Table 2 shows the most important phenotypic characteristics differentiating the novel strains from the other species of the genus *Campylobacter*. Bacterial cell shape was observed using a scanning electron microscope (Zeiss, LEO 420) following the protocol described by Kawamura *et al*. (2015). The organism exhibits a curved shape and cells became spherical or coccoid after 72h of incubation (Fig. 3).

In conclusion, the results from this taxonomic study clearly demonstrate that the isolates recovered from wild birds faecal samples comprise a novel species distinct from other currently known *Campylobacter* species, based on 16S rRNA, housekeeping genes, whole

genome sequence comparison, morphological, physiological and biochemical properties. The name *Campylobacter ornithocola* sp. nov. is proposed, with WBE38$^T$ (=CECT 9147$^T$ = LMG 29815$^T$) as the type strain.

**Description of *Campylobacter ornithocola* sp. nov.**

***Campylobacter ornithocola*** **[or.ni.tho'co.la. Gr. n. ornis, -ithos bird; L. suff. -cola (from L. n. incola) dweller; N.L. n. ornithocola bird dweller].**

Cells are Gram-negative curved rods, non-encapsulated, non-spore-forming and are 0.3–0.5 µm wide and 1.2–3 µm long. After incubation on Colombia agar (5% sheep blood) in microaerobic atmosphere at 37 °C for 48 h, colonies are glossy, slightly convex, round with smooth margins. Coccoid cells were observed in old cultures. Swarming on solid media was noted. Pigments are not produced. Grows on blood agar at 37 °C and at 42 °C under micro-aerobic culture conditions (does not require atmospheric hydrogen) and at 37°C in anaerobic conditions. No growth was observed at 37 °C on aerobiosis and at 25 °C under micro-aerobic conditions. No haemolysis is seen on blood agar. Catalase- and urease-activity is present but no oxidase. Esterase activity and reduction of Triphenyltetrazolium chloride (TTC) are variable.

Pathogenicity is unknown; strains have been recovered from faecal samples of wild birds. The type strain is WBE38$^T$ (=CECT 9147$^T$ = LMG 29815$^T$) which was isolated from a faecal sample in Valdivia, Chile.

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## Conflicts of interest

The authors have no conflict of interest to declare.

## Ethical statement

No experiments with humans or animals were carried out.

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**Table 1.** Average nucleotide identity based on BLAST (ANIb) values (in percentages) for *C. ornithocola* sp. nov. and the most closely related members of the genus *Campylobacter*

Strains: 1, *C. ornithocola* sp. nov. WBE38$^T$; 2, *C. lari* subsp. *concheus* LMG 11760; 3, *C. lari* subsp. *lari* RM 2100; 4, *C. subantarticus* LMG 24377$^T$; 5, *C. peloridis* LMG 23910$^T$; 6, *C. volucris* LMG 24379; 7, *C. insulaenigrae* NCTC 12927

| **Strains** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
|---|---|---|---|---|---|---|---|
| **1** | - | | | | | | |
| **2** | 90,8 | - | | | | | |
| **3** | 89,5 | 92,8 | - | | | | |
| **4** | 88,4 | 89,7 | 88,8 | - | | | |
| **5** | 86,0 | 86,2 | 86,1 | 85,3 | - | | |
| **6** | 82,0 | 82,3 | 83,1 | 81,8 | 82,4 | - | |
| **7** | 80,9 | 81,0 | 81,2 | 80,2 | 81,1 | 82,9 | - |

**Table 2.** Phenotypic characteristics that differentiate *Campylobacter ornithocola* sp. nov. from other *Campylobacter* species.

*Campylobacter* species: 1, *C. ornithocola* sp. nov (n = 17); 2, *C. avium*; 3, *C. canadensis*; 4, *C. coli*; 5, *C. concisus*; 6, *C. corcagiensis*; 7, *C. cuniculorum*; 8, *C. curvus;* 9, *C. fetus* subsp. *fetus*; 10, *C.fetus* subsp*. testudinum*; 11, *C. fetus* subsp. *venerealis*; 12, *C. geochelonis*; 13, *C. gracilis*; 14, *C. helveticus*; 15, *C. hepaticus;* 16, *C. hominis*; 17*, C. hyointestinalis* subsp. *hyointestinalis*; 18, *C. hyointestinalis* subsp. *lawsonii*; 19. *C. iguanorum*; 20, *C. insulaenigrae*; 21, *C. jejuni* subsp. *doylei*; 22, *C. jejuni* subsp. *jejuni*; 23, *C. lanienae*; 24, *C. lari* subsp. *concheus*; 25, *C. lari* subsp. *lari*; 26, *C. mucosalis*; 27, *C. peloridis*; 28, *C. rectus*; 29, *C. showae*; 30, *C. sputorum*; 31, *C. subantarticus*; 32, *C. upsaliensis*; 33, *C. ureolyticus*; 34, *C. volucris*. Data for reference taxa were taken from Foster et al., 2004; Matsuda and Moore, 2004; Debruyne et al., 2008, 2009; 2010a,b; Piccirillo et al., 2016; Van et al., 2016. +, 100% strains positive; -, 100% strains negative; (+), 80–94% strains positive; V, 42–66% strains positive; (-), 7–33% strains positive; ND, not determined.

| **Characteristic** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Oxidase | -* | + | + | + | V | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | V | + | + | + | + | + |
| Catalase | + | V | V | + | - | + | + | - | + | + | (+) | V | V | - | + | - | + | + | + | + | V | + | + | + | + | - | + | (-) | + | V | + | - | V | + |
| Urease | + | - | V | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | ND | - | - | - | - † | - | + | - † |
| Alkaline phosphatase | - | - | - | - | V | + | - | V | - | ND | - | - | - | - | ND | - | - | (-) | - | ND | - | - | + | - | - | (+) | ND | - | - | - | ND | - | - | - |
| **Reduction of** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Nitrate | V | + | V | + | (-) | (+) | + | + | + | + | (+) | + | (+) | + | V | - | + | + | + | + | - | + | + | + | + | (-) | ND | + | + | (+) | +‡ | + | + | + |
| 2,3,5, triphenyltetrazolium chloride (TTC) | (-) | - | ND | + | - | - | V | V | - | + | - | - | - | - | ND | - | - | - | ND | ND | V | + | ND | + | + | - | ND | - | - | - | ND | V | - | - |
| **Hydrolysis of** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Hippurate | - | + | - | - | - | - | - | (-) | - | - | - | + | - | - | (+) | - | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| Indoxyl acetate | - | + | - | + | - | V | + | V | - | - | - | - | V | + | + | - | - | - | - | - | + | + | - | ND | - | - | - | + | V | - | - | + | V | - |
| $H_2S$ production (TSI) | - | - | V | - | - | + | - | (-) | - | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - † | - | + | ND | - | V | + | - | - | - | - |
| α-Haemolysis | - | - | - | (-) | (-) | - | + | (-) | - | ND | V | - | - | + | - | - | V | V | - | ND | + | + | + | ND | + | - | ND | + | + | + | + | + | V | ND |
| **Growth at/in/on** | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 25°C (microaerobic) | - | - | - | - | - | ND | - | - | + | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| 37°C (microaerobic) | + | + | + | + | + | + | + | V | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | V | + | + | + | + | + |
| 42ºC (microaerobic) | + | + | + | + | (+) | + | (+) | V | (+) | V | - | - | V | + | + | (-) | + | + | + | - | - | + | + | + | + | + | + | (-) | V | + | + | + | V | + |
| 37°C (anaerobic) | + | - | + | - | + | + | - | + | (-) | + | V | + | + | - | - | + | - | + | + | - | - | - | + | ND | - | + | ND | + | + | + | + | - | V | + † |

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 37°C (aerobic) | - | - | - | - | - | - | - | - | - | V | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Glycine 1% | + | - | V | (+) | (+) | + | - | + | + | + | (-) | + | + | V | | + | + | V | + | + | (-) | + | - | (+) | + | V | + | + | V | + | (+) | + | + | - |
| $H_2$ requirement | - | V | - | - | + | - | - | + | - | - | - | - | + | - | - | + | V | V | - | ND | - | - | - | ND | - | + | ND | + | + | - | - | - | - | ND |
| DNA G+C (mol %) | 29.5 | 35 | ND | 31 | 37-41 | 31.9 | 32.4 | 45-46 | 33-35 | ND | 33-34 | 33.6 | 44-46 | 34 | 27.9 | 32.5 | 35-36 | 31-33 | 36 | ND | 31 | 30-31 | 36 | 30 | 29-30 | 36-38 | 29 | 45-46 | 44-46 | 29-33 | 30 | 32-36 | 28-30 | 29 |

* Oxidase negative test allows differentiating the new species from Urease-Positive Thermophilic *Campylobacter* (UPTC) strains.

† This test was determined for the corresponding type strain, in this study

‡ We obtained a different result from that published by Debruyne et al. (2010b), when we evaluated the nitrate test with the API Campy (nitrate reduction negative for strain LMG 24377$^T$).

355 **Figures legends**

356 **Fig. 1.** Cluster analysis based on the patterns obtained by ERIC-PCR from the
357 *Campylobacter ornithocola* sp. nov. strains.

358 **Fig. 2.** Neighbour-joining tree based on 16S rRNA sequences showing the phylogentic
359 position of *Campylobacter ornithocola* sp. nov. within the genus *Campylobacter*. Bootstrap
360 values >70%, generated from 500 replicates, are shown at the nodes. The scale bar
361 represents substitutions per site.

362 **Fig. 3.** Images of cells of strain WBE38$^T$ as observed with scanning electron microscopy.
363 The organism exhibits a curved shape while spherical or coccoid forms can also be
364 observed. Bars, 3 µm.

Figure 1

Click here to download Figure Fig. 1 (ERIC-PCR).pptx

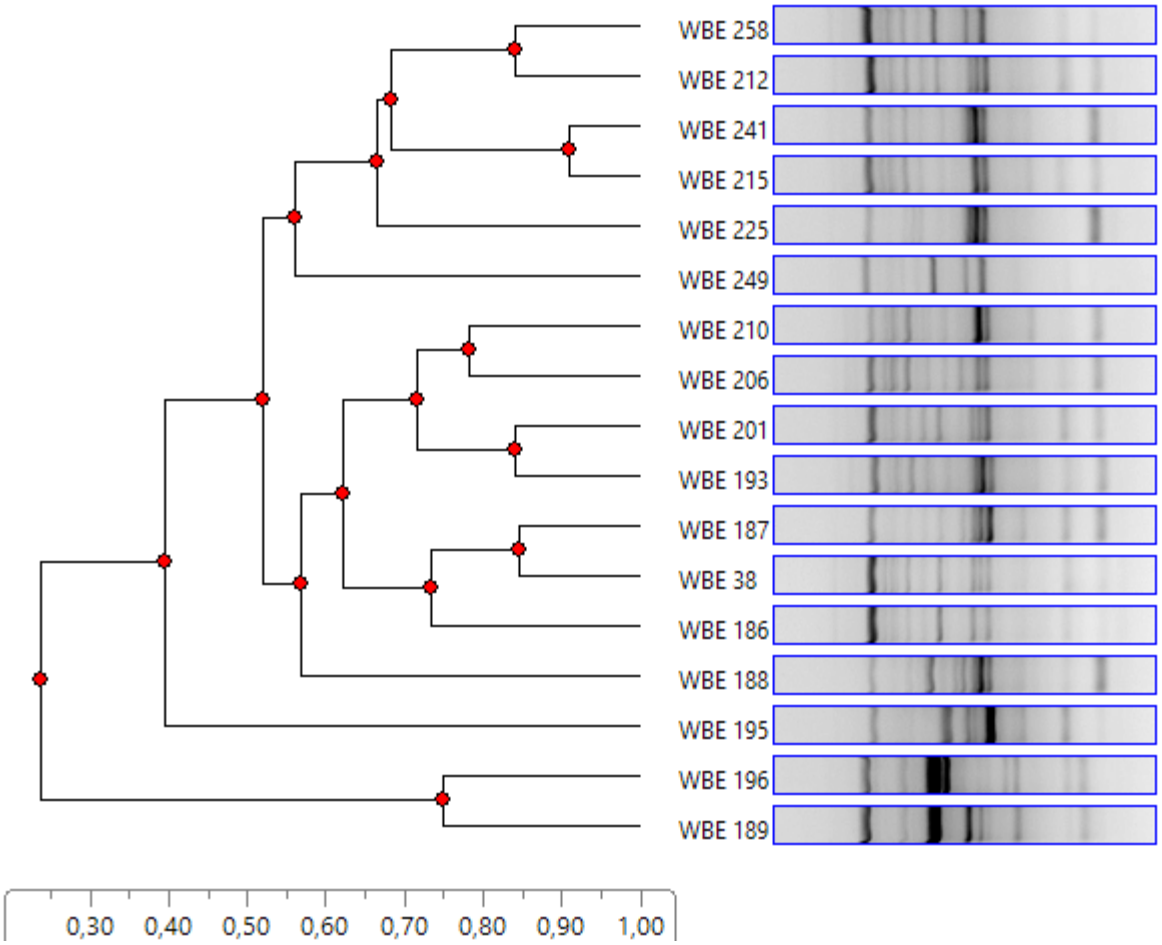


Figure 2

Click here to download Figure Fig. 2 (16S rRNA).pptx

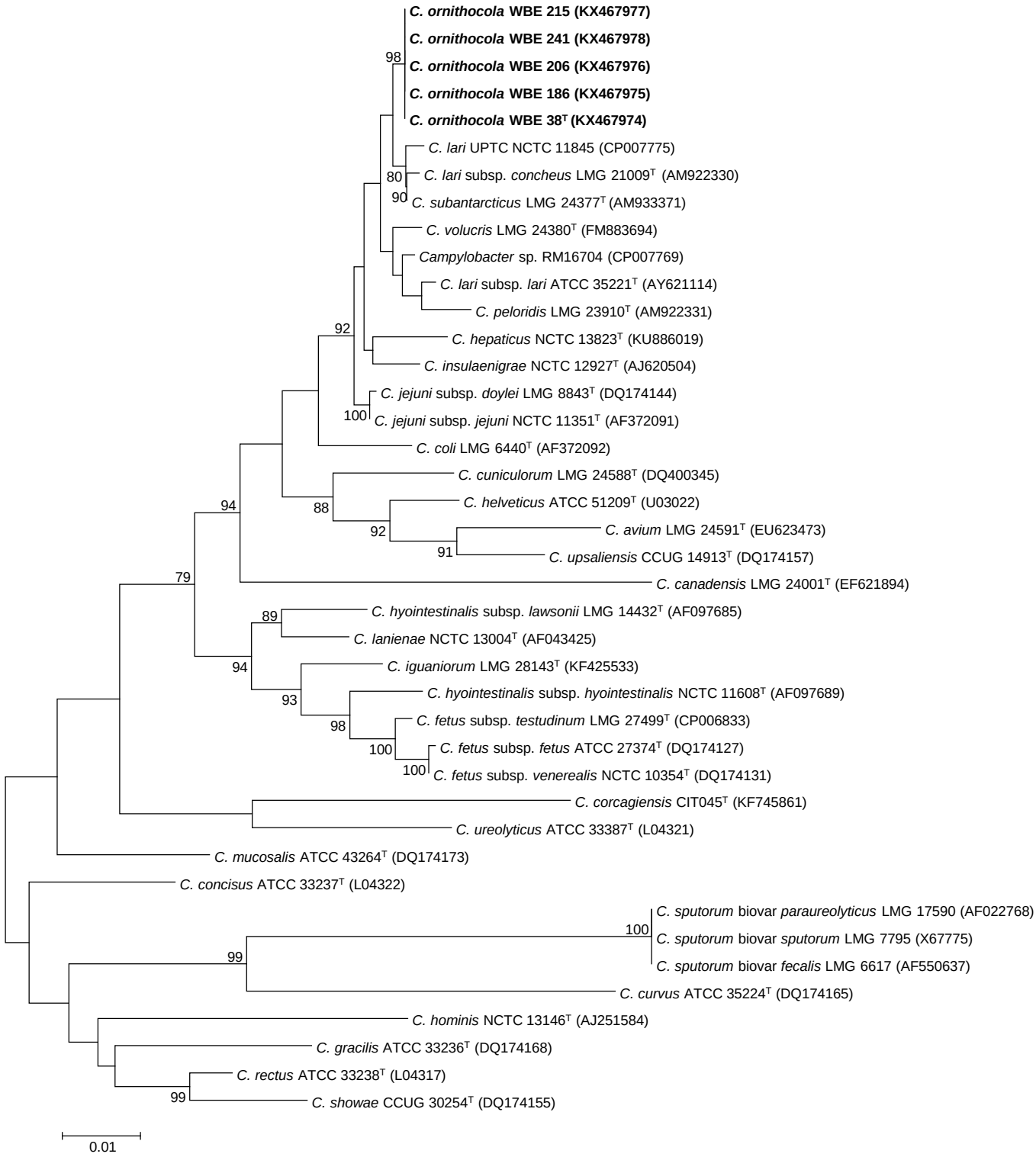
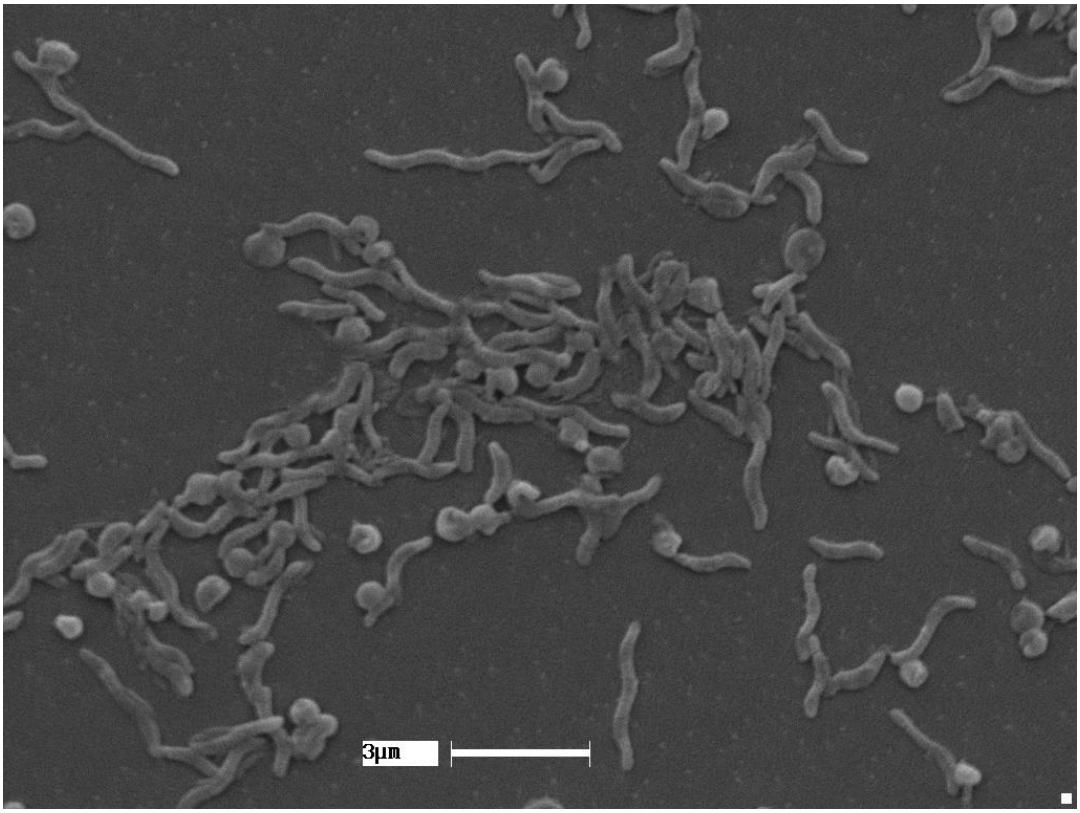


Figure 3

Click here to download Figure Fig. 3 (SEM).pptx



Supplementary data on-line

International Journal of Systematic and Evolutionary Microbiology

# *Campylobacter ornithocola* sp. nov., a new member of the *Campylobacter lari* group isolated from wild bird faecal samples

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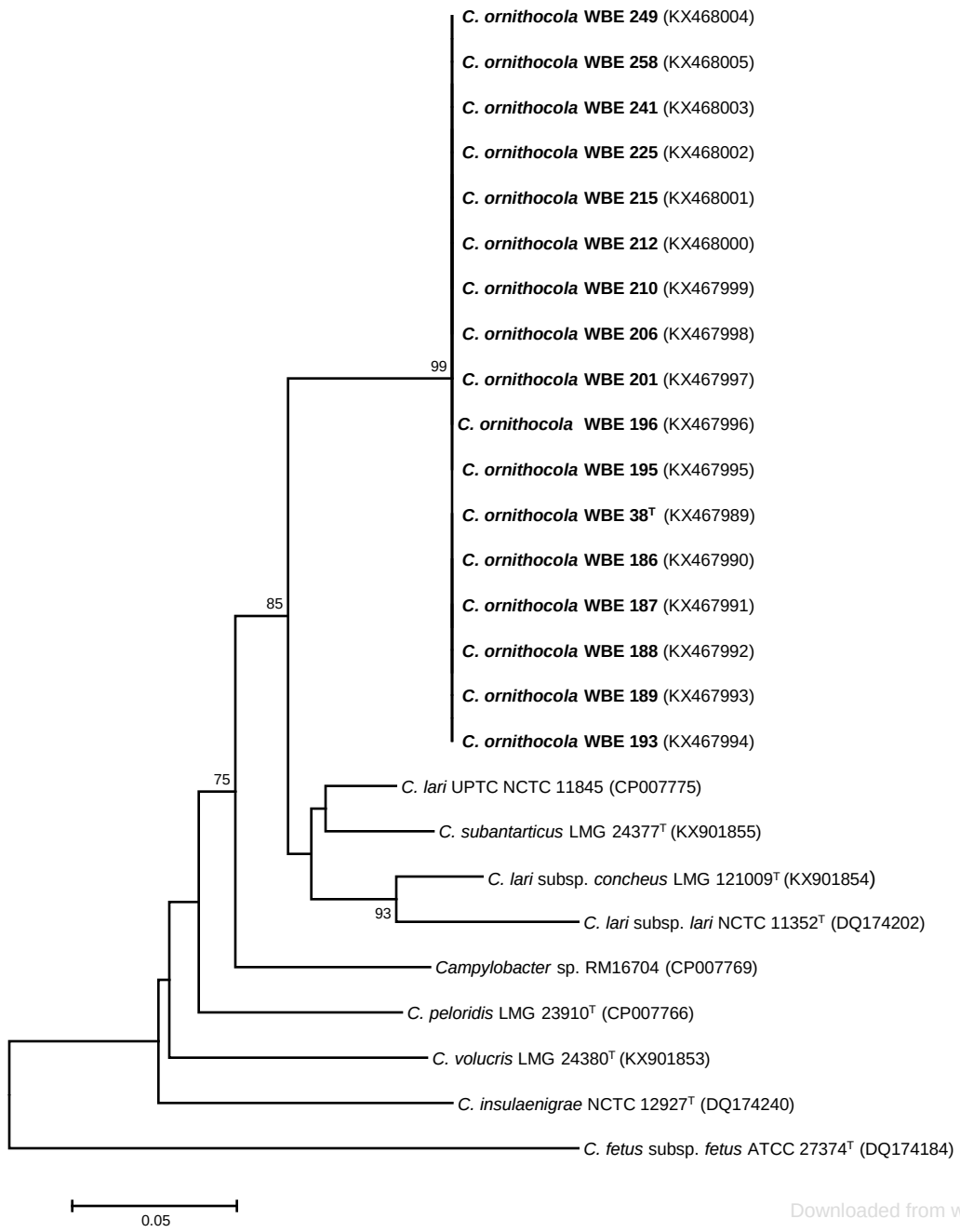
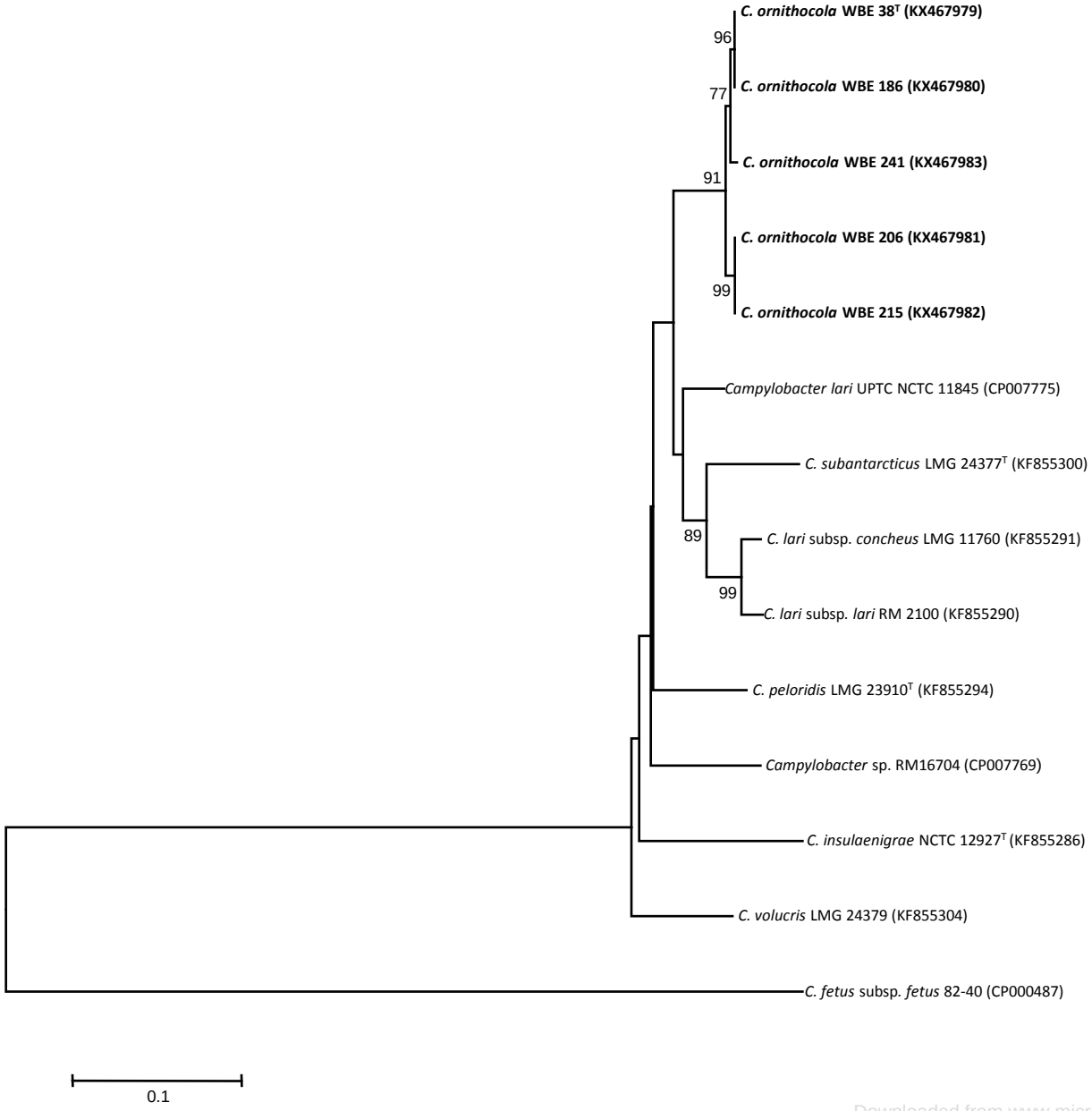
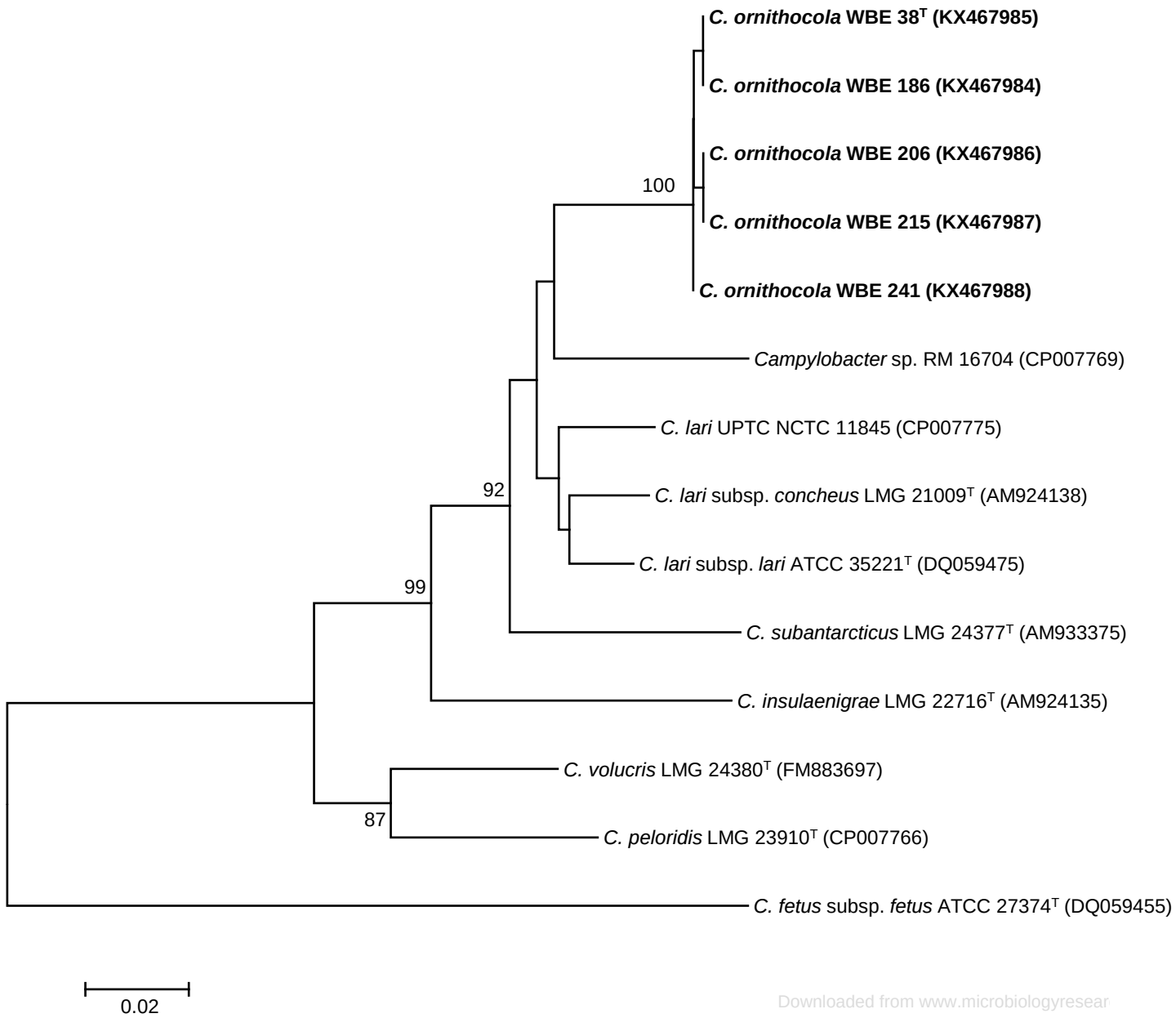



**Supplementary Fig. 1.** Neighbour-joining tree based on *rpoB* sequences showing the phylogentic position of *Campylobacter ornithocola* sp. nov. within *Campylobacter lari* group. Bootstrap values >70%, generated from 500 replicates, are shown at the nodes. The scale bar represents substitutions per site.




**Supplementary Fig. 2.** Neighbour-joining tree based on *atpA* sequences showing the phylogentic position of *Campylobacter ornithocola* sp. nov. within *Campylobacter lari* group. Bootstrap values >70%, generated from 500 replicates, are shown at the nodes. The scale bar represents substitutions per site.




**Supplementary Fig. 3.** Neighbour-joining tree based on *cpn60* sequences showing the phylogentic position of *Campylobacter ornithocola* sp. nov. within *Campylobacter lari* group. Bootstrap values >70%, generated from 500 replicates, are shown at the nodes. The scale bar represents substitutions per site.