Campylobacter subantarcticus sp. nov., isolated from birds in the sub-Antarctic region

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Short title: *Campylobacter subantarcticus* sp. nov.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of LMG 24377^T, LMG 24374, LMG 24375 and LMG 24378 are AM933371, AM933372, AM933373 and AM933374, respectively. The GenBank/EMBL/DDBJ accession numbers for *hsp60* gene sequences of LMG 24377^T and LMG 24374 are AM933375 and AM933376, respectively.

Abstract

Six Gram-negative, spiral-shaped, micro-aerobic isolates were obtained during a sampling from wild birds in the sub-Antarctic region. Based on initial observations, these isolates were classified as '*Campylobacter lari*-like'. Further characterization was performed by a polyphasic approach, including whole-cell protein and amplified fragment length polymorphism (AFLP) analysis, 16S rRNA and *hsp60* gene sequencing, biochemical analysis, and DNA-DNA hybridizations.

Here, we present comprehensive phylogenetic, genomic and phenotypic evidence that these *C. lari*-like isolates represent a novel species within the genus *Campylobacter*, for which the name *Campylobacter subantarcticus* sp. nov. is proposed. The type strain is R-3023^T (=CCUG 38513^T=LMG 24377^T).

The genus *Campylobacter* (Sebald & Veron, 1963) presently comprises 20 validly named species, and 8 subspecies, with species found in both man and a wide range of domestic and wild animals and birds. Species most often associated with captive or free-living wild birds, either asymptomatic or with disease symptoms, include *Campylobacter lari* subsp. *lari* and *Campylobacter jejuni* subsp. *jejuni*, *Campylobacter coli*, and urease positive thermophilic *Campylobacter* (UPTC) *lari* isolates (Waldenstrom *et al.*, 2002; Waldenstrom *et al.*, 2007). The more recently named species *Campylobacter canadensis* has exclusively been isolated from captive whooping cranes (Inglis *et al.*, 2007). The presence of zoonotic species in wild birds may provide a reservoir for human-pathogenic species, either through direct contact or through contamination of the environment.

During a sampling of wild birds and fur seals at Bird Island (54° 00' S, 38° 02' W) in the South Georgian archipelago in 1996, a collection of *Campylobacter* isolates was obtained. Several of these isolates were initially designated *C. lari*-like, based on biochemical similarities. Six of these isolates were included in the present polyphasic taxonomic study: three were isolated from grey headed albatrosses (*Diomedea chrysostoma*), two from black browed albatrosses (*D. melanophris*) and one from a gentoo penguin (*Pygoscelis papua*). No isolates could be obtained from Antarctic fur seals, suggesting that this *Campylobacter* species is restricted to birds. Strains were examined by whole-cell protein SDS-PAGE, AFLP, 16S rRNA and *hsp60* gene sequencing. Phenotypic characteristics were determined, and relevant DNA-DNA hybridisations were performed.

In February / March 1996 fecal swabs were taken from 10 adult female and 40 female Antarctic fur seal pups (*Arctocephalus gazella*), 30 adult gentoo penguins, 50 macaroni penguin chicks (*Eudyptes chrysolophus*), 50 black browed albatross chicks and 50 grey headed albatross chicks. Fecal samples were collected using cotton wool swabs inserted into the rectum or cloaca. Samples were stored in a charcoal transport medium (Transwab, BioDisc, Solna, Sweden) at 5 – 10°C and transported to Sweden within three weeks. Samples were plated on *Campylobacter* selective medium (42.5 g/l Columbia Agar Base,

Becton Dickinson, Cockeysville, Maryland, USA, 5 % citrated horse blood, 10 mg/l Vancomycin, 2500 IE/l Polymyxin B, 5 mg/l Trimetoprim) and incubated for 48 h at 42°C under microaerobic conditions. Colonies showing a Gram-negative seagull-like cell morphology under light microscopy where sub-cultured onto blood agar plates. Samples were stored at -80°C in Trypticase Soy Broth supplemented with 15 % glycerol.

Strains were cultured on Mueller-Hinton agar supplemented with 5% horse blood at 37°C for 48h in microaerobic conditions (approx. 4% O_2, 6.5% CO_2, 6.5% H_2, 83% N_2). DNA was extracted as described by Pitcher *et al.* (1989).

Protein extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Pot *et al.* (1994). For whole-cell protein SDS-PAGE analysis, similarity of the obtained normalized SDS-PAGE patterns was determined by the Pearson product moment correlation coefficient, after which clustering was performed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), using BioNumerics version 4.61 (Applied Maths, Belgium). For numerical analysis a variable dense band region (36.1 – 43.2 kDa) (Vandamme *et al.*, 1990) was excluded to increase species discrimination. The results of the numerical analysis, in combination with visual inspection of the SDS-PAGE patterns, demonstrated that the SDS-PAGE patterns of the novel species were distinct from those of *C. lari*, and all other known *Campylobacter* species (data not shown).

Amplified Fragment Length Polymorphism (AFLP) analysis was performed as described by Debruyne *et al.* (in press). After normalization, the obtained AFLP profiles were included in an in-house AFLP reference database, containing profiles from type and reference strains of all established *Campylobacter* species. The similarity between profiles was determined by the Pearson correlation coefficient, and cluster analysis was performed by UPGMA, using BioNumerics v 4.61. AFLP profiles from the six strains representing the novel species were divergent from those of strains of other *Campylobacter* species, and formed a distinct cluster (Fig. 1).

To support the delineation of the groups defined by the above genomic and proteomic analyses, phenotypic testing was performed. Tests included were evaluation of growth on media containing 1.0% glycine, 0.02% safranin, nalidixic acid (32 mg l^{-1}), cephalothin (32 mg l^{-1}), metrodinazole (4 mg l^{-1}), carbenicillin (32 mg l^{-1}) and 0.1% sodium deoxycholate. Growth on MacConkey agar and unsupplemented nutrient agar (Oxoid no. 2) were also evaluated, as were catalase activity, hippurate hydrolysis, H_2S production on TSI agar, growth at 42°C and α-haemolysis. Methods for biochemical testing were as described previously (On & Holmes, 1991a; 1991b; 1992). Differentiating characteristics are listed in Table 1.

To determine the phylogenetic position of the novel species, 16S rRNA gene sequences of the strains LMG 24374, LMG 24375, LMG 24377^T and LMG 24378 (randomly selected) were determined as described previously (Vandamme *et al*., 2006). Sequences were assembled using BioNumerics v 5.1. Comparison by the FASTA algorithm to the EMBL sequence database revealed that the nearest phylogenetic neighbours were *C. lari* subsp. *concheus*, *C. lari* subsp. *lari*, *C. jejuni*, *C. coli*, *C. insulaenigrae* and *C. peloridis*, all with similarity levels exceeding 97%. Strains LMG 24375, LMG 24377^T and LMG 24378 had identical 16S rRNA gene sequences (100% sequence similarity), while LMG 24374 was slightly more divergent (99.5%). Sequences were aligned using the ClustalX software package (Thompson *et al.*, 1997), and clustering was performed by the neighbor-joining method (Saitou & Nei, 1987) using BioNumerics v 5.1. Unknown bases were discarded for the analysis. Bootstrap values were determined using 500 replicates (Fig. 2). Polymorphisms within the 16S rRNA gene were inadequate to distinguish among the novel taxon and *C. lari* subsp. *concheus*, with interspecies sequence similarities (99.4-99.9%) being equal to or exceeding intraspecies sequence similarities (99.5-100%). To improve species discrimination, partial *hsp60* gene sequences of LMG 24374 and LMG 24377^T were determined as described before (Debruyne et al., in press). Kärenlampi *et al*. (2004) demonstrated that phylogeny based on the *hsp60* gene sequence, coding for the 60 kDa heat shock protein, was similar to that of the 16S rRNA gene. However, *hsp60* was found to provide a better resolution for *Campylobacter*

137 species, with lower interspecies sequence similarities and high intraspecies sequence
138 similarities. Pairwise comparison of *hsp60* gene sequences from the novel taxon and from *C.*
139 *lari* subsp. *concheus* demonstrated a clear separation between intraspecies (100%) and
140 interspecies (93.3-93.9%) sequence similarities, making species discrimination feasible (Fig.
141 3).

142 For the determination of G+C content, DNA was enzymically degraded into nucleosides as
143 described by Mesbah & Whitman (1989). The nucleoside mixture was separated by HPLC
144 using a Waters SymmetryShield C8 column maintained at 37 °C. The solvent was 0.02 M
145 $(NH_4)H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was
146 used as the calibration reference. The DNA G+C contents of the strain LMG 24377^T was
147 30%, which falls within the range reported for genus *Campylobacter*, i.e. 29-47%.

148 DNA-DNA hybridisations were performed between strain LMG 24377^T and type strains of its
149 closest relatives, i.e. *C. lari* subsp. *lari*, *C. lari* subsp. *concheus*, *C. peloridis*, *C. jejuni* subsp.
150 *jejuni*, *C. coli* and *C. insulaenigrae*. DNA was extracted from 0.25–0.5 g (wet wt) cells as
151 described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed with
152 photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio
153 Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization
154 temperature was 30 °C. Reciprocal experiments were performed for every pair of strains and
155 standard deviation values ranged from 0.7-7.5. DNA–DNA hybridisation values between
156 strain LMG 24377^T and the type strain of *C. lari* subsp. *lari* (LMG 8846^T), *C. lari* subsp.
157 *concheus* (LMG 21009^T), *C. peloridis* (LMG 23910^T), *C. jejuni* subsp. *jejuni* (LMG 8841^T), *C.*
158 *coli* (LMG 6440^T), and *C. insulaenigrae* (LMG 22716^T) were 57, 55, 38, 21, 16 and 41%,
159 respectively. All these values are well below the threshold of 70% for species delineation
160 (Stackebrandt & Goebel, 1994).

161 The present study demonstrates that the six bird isolates represent a novel species within
162 the genus *Campylobacter* which can be distinguished from other *Campylobacter* species by

whole cell protein electrophoresis, AFLP fingerprinting, *hsp60* gene sequence analysis and biochemical characteristics. Below we formally propose to classify these strains as *Campylobacter subantarcticus* sp. nov., with LMG 24377^T (=CCUG 38513^T) as the type strain.

Description of Campylobacter subantarcticus sp. nov.

Campylobacter subantarcticus [sub.ant.arc'ti.cus N.L. masc. adj. *subantarcticus*], pertaining to the sub-Antarctic region, from where the organism was isolated.

Cells are slightly curved, Gram negative rods. Colonies are colourless, round, entire, convex, 1-1.5 mm in diameter after culture on 5% blood agar for 72h under microaerobic conditions. Oxidase and catalase positive, strains do not hydrolyse hippurate, and no production of H_2S on TSI agar. Growth at 42°C under micro-aerobic conditions. Growth on media containing 32 mg ml^{-1} nalidixic acid, and most strains grow on media containing 1% glycine. Most strains do not grow on media containing 4 mg ml^{-1} metrodinazole or on MacConkey agar. No growth observed on unsupplemented nutrient agar, and on media containing 0.02% safranin, 0.1% sodium deoxycholate, 32 mg ml^{-1} cephalothin or 32 mg ml^{-1} carbenicillin. Alpha-haemolysis observed on 5% blood agar

Pathogenicity unknown. Strains have been recovered from wild birds in the sub-Antarctic region. The type strain is R-3023^T (=LMG 24377^T=CCUG 38513^T), which was isolated from a grey headed albatross in 1996.

Acknowledgements

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Figure legends:

Figure 1: Dendrogram representing the AFLP fingerprints of six strains representing the novel species C. subantarcticus sp. nov. and selected Campylobacter reference strains. Similarity was determined by the Pearson product moment correlation coefficient and clustering was performed by UPGMA.

Figure 2: Phylogenetic tree based on 16S rRNA gene sequences constructed by the neighbor-joining method. Bootstrap values (%) are indicated at the nodes.

Figure 3: Neighbor-joining tree based on partial hsp60 gene sequences. All sequences are 555 bp in length, with the exception of the sequence for C. cuniculorum, which is 489 bp in lengtj. Bootstrap values (%) are indicated at the nodes.

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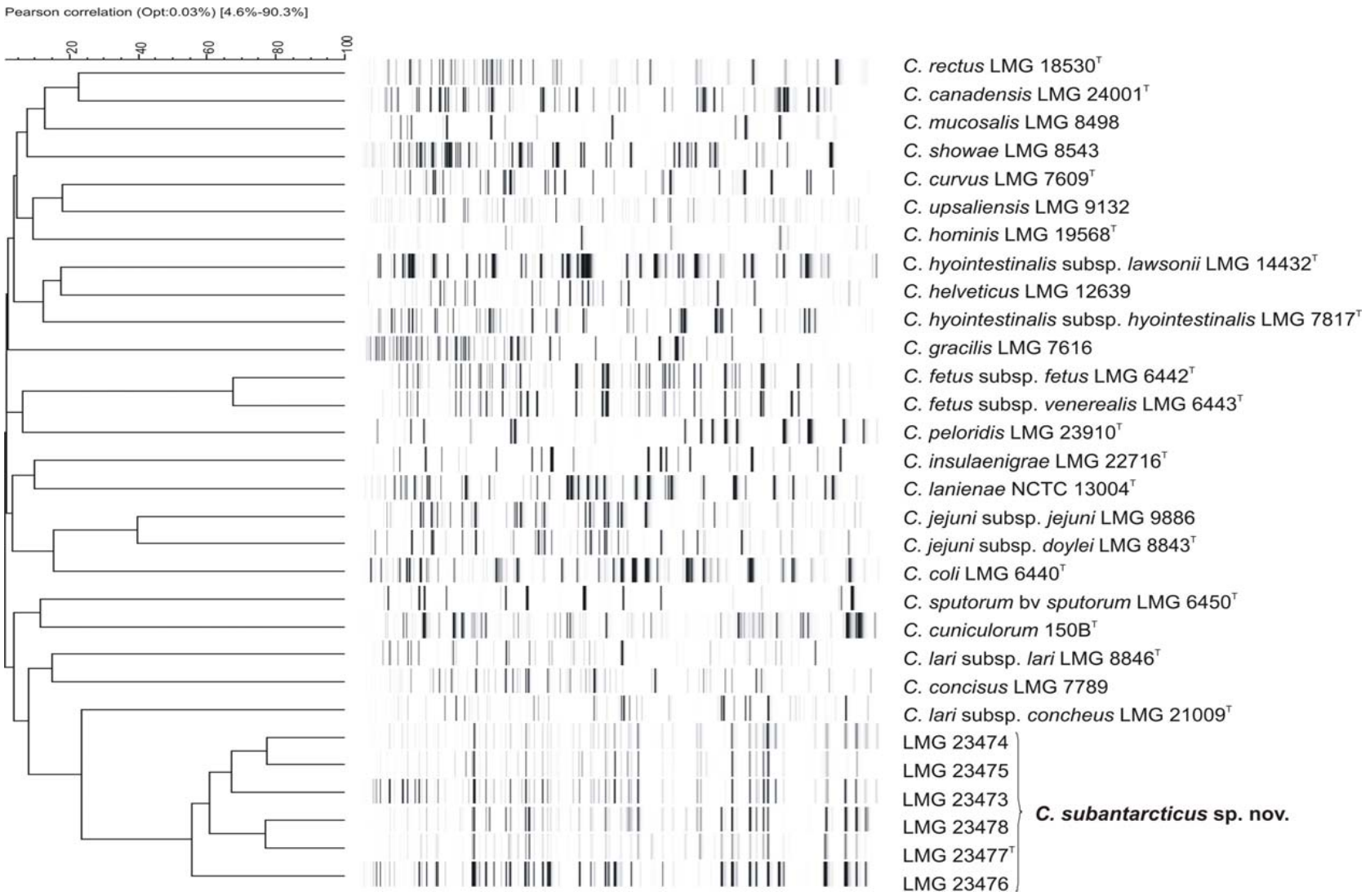
Table 1: Differentiating phenotypic characteristics. 1, *C. subantarcticus* sp. nov. (n=6); 2, *C. canadensis*; 3, *C. coli*; 4, *Campylobacter concisus*; 5, *Campylobacter cuniculorum*; 6, *Campylobacter curvus*; 7, *Campylobacter fetus* subsp. *fetus*; 8, *C. fetus* subsp. *venerealis*; 9, *Campylobacter gracilis*; 10, *Campylobacter helveticus*; 11, *Campylobacter hyointestinalis*; 12, *Campylobacter hominis*; 13, *C. insulaenigrae*; 14, *C. jejuni*; 15, *Campylobacter lanienae*; 16, *C. lari* subsp. *concheus*; 17, *C. lari* subsp. *lari*; 18, *Campylobacter mucosalis*; 19, *C. peloridis*; 20, *Campylobacter rectus*; 21, *Campylobacter showae*; 22, *Campylobacter sputorum*; 23, *Campylobacter upsaliensis*. +: all strains positive; -: all strains negative; (+): 80-94% strains positive; (-): 5-33% strains positive; V: 35-67% positive. Additional data for reference species were taken from Inglis *et al.* (2007), Lawson *et al.* (2001), On *et al.* (1996) and Zanoni *et al.* (in press).

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Catalase	+	V	+	-	+	-	+	(+)	(-)	-	+	-	+	(+)	+	+	+	-	+	(-)	+	V	+
Hippurate hydrolysis	-	-	-	-	-	(-)	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
H_2S production (TSI)	-	V	-	(-)	-	(-)	-	-	-	-	-	(+)	-	-	-	ND	-	+	ND	-	V	+	-
Growth at 42°C	+	+	+	(+)	(+)	V	(+)	-	(-)	+	+	V	-	V	+	+	+	+	+	(-)	V	(+)	+
Alpha-haemolysis	+	-	(-)	(-)	+	(-)	-	(-)	-	+	V	-	+	(+)	+	ND	+	(-)	ND	+	+	+	+
MacConkey agar	(-)	-	V	-	-	(+)	(+)	V	(+)	-	V	-	-	(-)	+	+	(+)	(+)	+	-	+	V	-
Nutrient agar	-	-	+	(-)	+	+	+	(+)	+	(+)	+	+	V	+	-	+	+	+	+	(-)	V	(+)	+
Glycine (1%)	(+)	V	(+)	(-)	-	+	+	(-)	+	V	V	+	-	V	-	+	+	V	+	+	V	+	+
Safranin (0.02%)	-	ND	+	(-)	ND	+	+	(+)	+	-	+	-	-	V	-	-	+	+	-	-	-	(+)	+

Sodium deoxycholate (0.1%)	-	ND	+	(-)	ND	(+)	+	(+)	(+)	(-)	V	-	+	V	-	V	+	-	V	-	-	V	V
Nalidixic acid (32 mg L^{-1})	+	V	-	(+)	V	+	+	V	V	-	+	(+)	+	-	+	-	(+)	(+)	(+)	(+)	-	(+)	-
Cephalothin (32 mg L^{-1})	-	-	+	-	(+)	-	-	-	-	-	(-)	-	+	V	+	+	+	V	(-)	-	-	-	(-)
Metrodinazole (4 mg L^{-1})	(-)	ND	(+)	(-)	ND	-	(+)	V	-	V	V	-	+	V	+	+	+	(+)	+	-	+	(-)	(+)
Carbenicillin (32 mg L^{-1})	-	ND	(+)	-	ND	-	-	-	-	V	-	-	+	V	+	+	+	-	-	-	-	-	-


Pearson correlation (Opt:0.03%) [4.6%-90.3%]
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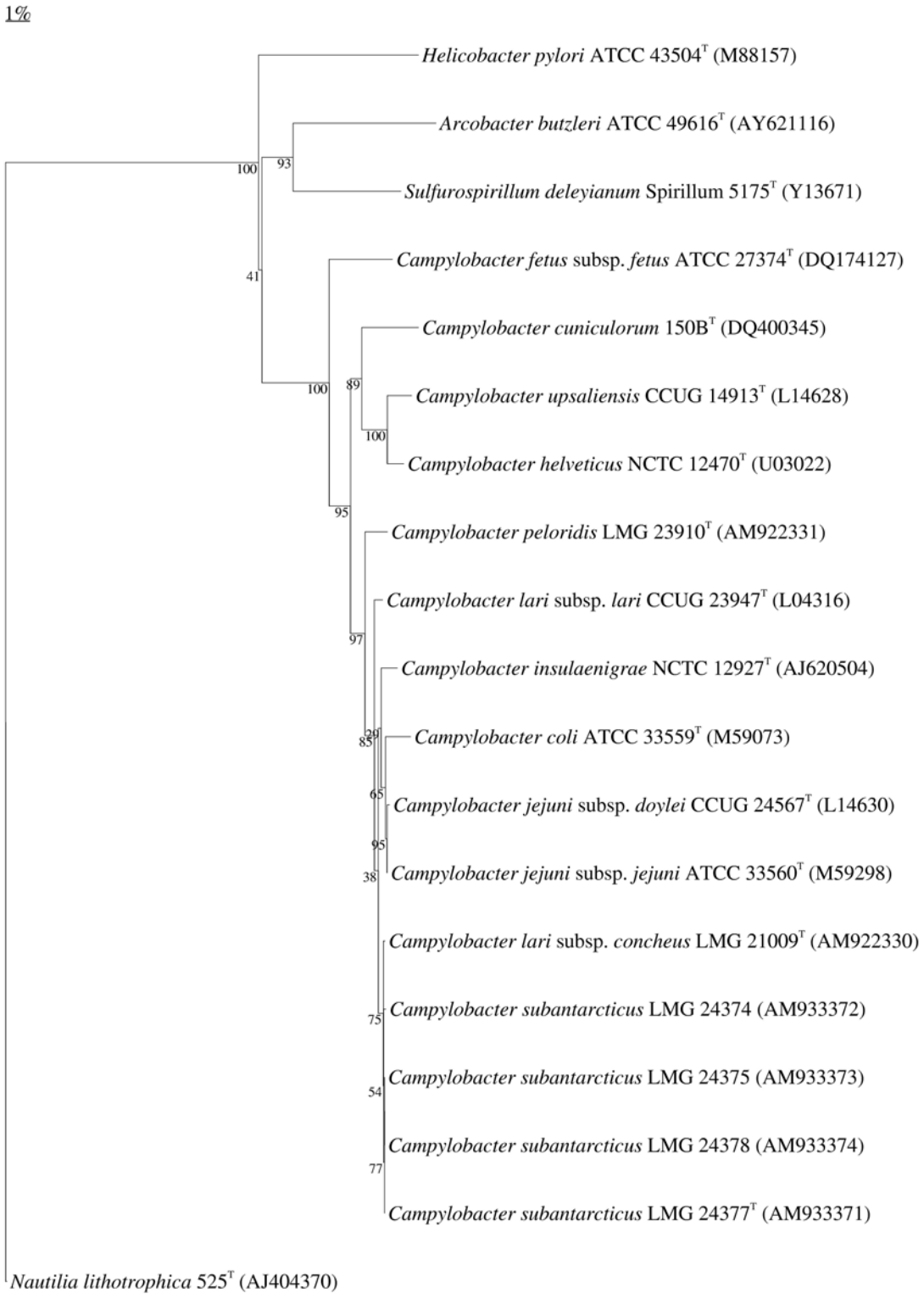
C. rectus LMG 18530T
C. canadensis LMG 24001T
C. mucosalis LMG 8498
C. showae LMG 8543
C. curvus LMG 7609T
C. upsaliensis LMG 9132
C. hominis LMG 19568T
C. hyointestinalis subsp. lawsonii LMG 14432T
C. helveticus LMG 12639
C. hyointestinalis subsp. hyointestinalis LMG 7817T
C. gracilis LMG 7616
C. fetus subsp. fetus LMG 6442T
C. fetus subsp. venerealis LMG 6443T
C. peloridis LMG 23910T
C. insulaenigrae LMG 22716T
C. lanienae NCTC 13004T
C. jejuni subsp. jejuni LMG 9886
C. jejuni subsp. doylei LMG 8843T
C. coli LMG 6440T
C. sputorum bv sputorum LMG 6450T
C. cuniculorum 150BT
C. lari subsp. lari LMG 8846T
C. concisus LMG 7789
C. lari subsp. concheus LMG 21009T
LMG 23474
LMG 23475
LMG 23473
LMG 23478
LMG 23477T
LMG 23476
C. subantarcticus sp. nov.

Fig 1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Campylobacter cuniculorum 150BT (DQ400345)
Campylobacter upsaliensis CCUG 14913T (L14628)
Campylobacter helveticus NCTC 12470T (U03022)
Campylobacter peloridis LMG 23910T (AM922331)
Campylobacter lari subsp. lari CCUG 23947T (L04316)
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Campylobacter jejuni subsp. doylei CCUG 24567T (L14630)
Campylobacter jejuni subsp. jejuni ATCC 33560T (M59298)
Campylobacter lari subsp. concheus LMG 21009T (AM922330)
Campylobacter subantarcticus LMG 24374 (AM933372)
Campylobacter subantarcticus LMG 24375 (AM933373)
Campylobacter subantarcticus LMG 24378 (AM933374)
Campylobacter subantarcticus LMG 24377T (AM933371)
Nautilia lithotrophica 525T (AJ404370)
100
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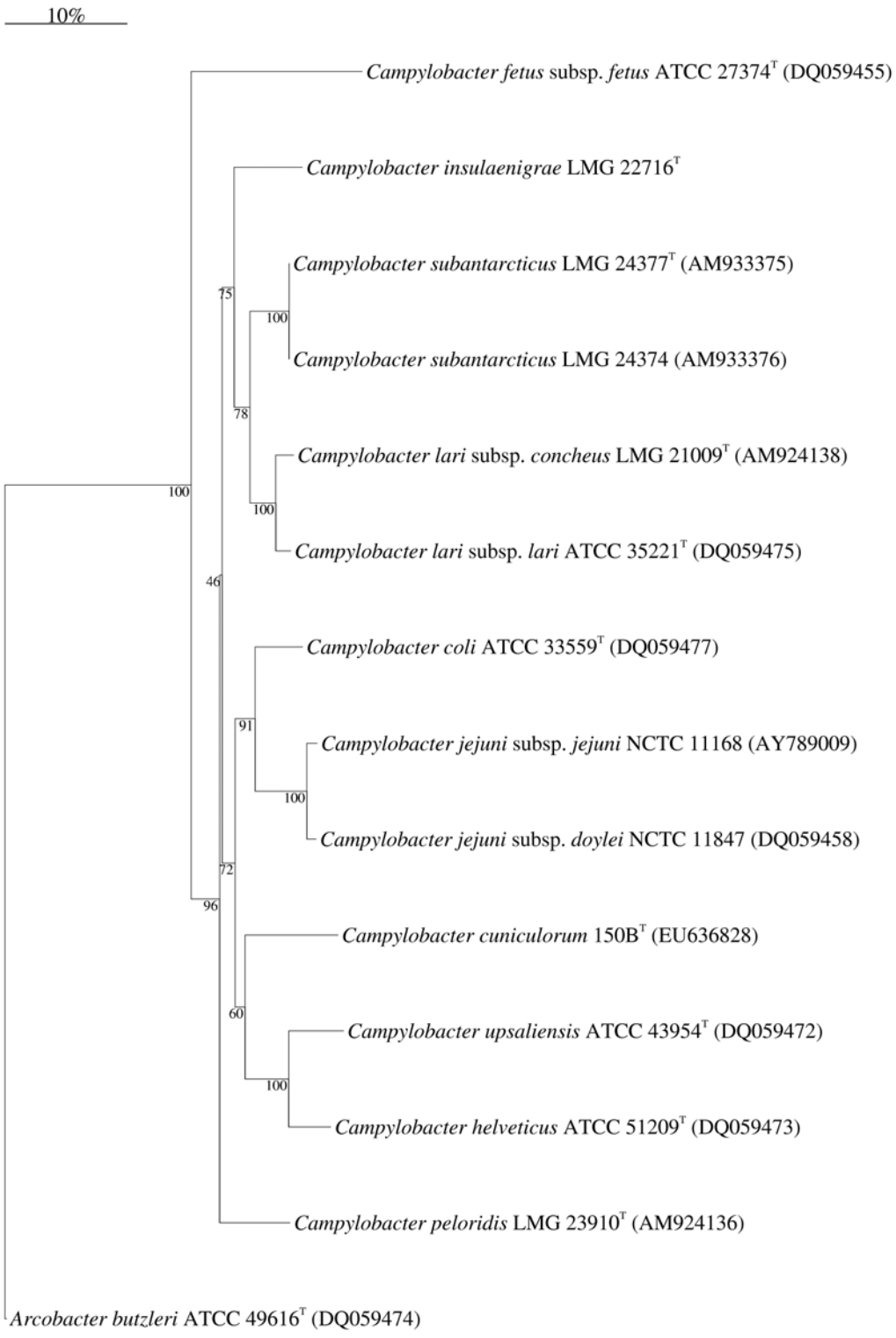
272 **Fig 2**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Campylobacter lari subsp. concheus LMG 21009T (AM924138)
Campylobacter lari subsp. lari ATCC 35221T (DQ059475)
Campylobacter coli ATCC 33559T (DQ059477)
Campylobacter jejuni subsp. jejuni NCTC 11168 (AY789009)
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Arcobacter butzleri ATCC 49616T (DQ059474)
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100
72
96
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Fig 3