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CAMPYLOBACTER VULPIS SP. NOV. ISOLATED FROM WILD RED FOXES

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1 **ABSTRACT**

2 During a sampling of wild red foxes (*Vulpes vulpes*) for the detection of
3 Epsilonproteobacteria, 14 strains were isolated from the caecal contents of 14
4 epidemiologically-unrelated animals. A genus-specific PCR indicated that the isolates
5 belonged to the genus *Campylobacter*. Based on the results of a species-specific PCR, the
6 isolates were initially identified as *C. upsaliensis*. However, multi-locus sequence typing
7 (MLST) revealed that the isolates were significantly different from the *C. upsaliensis* present
8 in the MLST database. A polyphasic study, including conventional biochemical and tolerance
9 characteristics, morphology by transmission electron microscopy (TEM), MALDI-TOF
10 analysis, and genetic comparisons based on partial 16S rDNA and *atpA* gene sequences, was
11 undertaken. Finally, the complete genome sequence of the type strain 251/13^T and the draft
12 genome sequences of the other isolates were determined. Average nucleotide identity,
13 average amino acid identity and *in silico* DNA-DNA hybridization analyses confirmed that
14 the isolates represent a novel taxon for which the name *Campylobacter vulpis* sp. nov. is
15 proposed, with isolate 251/13^T (= CCUG 70587^T = LMG 30110^T) as the type strain. In order
16 to allow a rapid discrimination of *C. vulpis* from the closely-related *C. upsaliensis*, a specific
17 PCR test was designed, based on *atpA* gene sequences.

18

19

20 **Keywords**: *Campylobacter vulpis* sp. nov., polyphasic taxonomic study, red foxes, *Vulpes*
21 *vulpes*.

INTRODUCTION

The genus *Campylobacter* currently contains 37 validly-described taxa. Many of these taxa have been recovered from a variety of terrestrial and marine mammals, reptiles, and both domesticated and wild birds [21]. *Campylobacter* strains are routinely isolated from the fecal samples and rectal swabs of domestic dogs, both healthy and diarrheic [1, 19]. One of the primary *Campylobacter* colonizers of domestic dogs is *Campylobacter upsaliensis* [1, 21]. The related species, *Campylobacter helveticus* [47], is also recovered from domestic dogs, although at a lower frequency [4, 14]. In addition to *C. upsaliensis* and *C. helveticus*, *C. jejuni, C. coli, C. lari* and *C. hyointestinalis* also colonize domestic dogs [19], along with at least nine other *Campylobacter* species (i.e., *C. showae, C. concisus, C. fetus, C. gracilis, C. mucosalis, C. rectus, C. sputorum, C. ureolyticus* and *C. volucris*) at a lower reported frequency [11, 19], suggesting that domestic dogs are host to a wide variety of campylobacters. Several of these emerging campylobacters have also been isolated from human clinical samples [1, 21, 29]. Thus, their presence in domestic dogs is a potential human health concern due to the close contact of dogs and humans. Most of the reported canine-associated epidemiology of *Campylobacter* pertains to pet and sheltered animals. Therefore, data on the prevalence of *Campylobacter* within wild canid populations is limited, although *C. upsaliensis* has been recovered from coyotes [33].

In 2013, a survey on the prevalence of *Campylobacter* spp. was carried out on wild red foxes (*Vulpes vulpes*) shot during population control programs in different municipalities of the Province of Bologna. Fourteen isolates of Gram-negative, spiral-shaped cells resembling members of the genus *Campylobacter* were recovered from the caecal contents of an equal number of epidemiologically-unrelated wild red foxes. In this study, these isolates were characterized through biochemical tests and by genomic analyses and were determined to comprise a distinct and novel taxon within the genus *Campylobacter*.

47

48 **MATERIALS AND METHODS**

49 *Isolation and culturing*

50 From January to July 2013 the caecal contents collected from a total of 45 wild red foxes (26
51 females and 19 males, 12 subadults <1 year and 33 adults) were examined for *Campylobacter*
52 spp. and *Helicobacter* spp. Foxes were shot during population control programs in the
53 province of Bologna. For this survey, only animals dead ≤12 hours and kept at refrigeration
54 temperature until necropsy were selected. Bacterial strain isolations were performed on
55 Nutrient sheep-blood agar [Nutrient Broth No 2 (Oxoid) amended with 1.5% (w/v) Bacto
56 Agar (Difco) and 5% (v/v) sheep blood], using the filter technique of Steele and McDermott
57 that was modified as previously described [52], and on selective media [Campylobacter
58 Selective Agar (Skirrow) and Campylobacter CAT Agar (Oxoid)]. Plates were incubated at
59 37 ± 1°C for 72 h in a microaerobic atmosphere, with hydrogen, obtained by the gas
60 replacement method, using an anaerobic gas mixture (10% H_2, 10% CO_2, 80% N_2) as
61 described [5]. Pure cultures were obtained after dilution and repeated sub-culturing onto
62 plates of Nutrient sheep-blood agar never older than 7 days.

63

64 *Preliminary taxonomic identification and MLST typing*

65 Genomic DNA was purified using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich,
66 St. Louis, MO, USA) and amplified using a *Campylobacter* genus-specific PCR [28] and a
67 species-specific PCR for *C. upsaliensis* and *C. helveticus* as previously described [27]. Based
68 on a preliminary identification of *C. upsaliensis*, the isolates were typed by multi-locus
69 sequence typing (MLST) according to published protocols [31], with the PCR amplifications
70 performed as described elsewhere (https://pubmlst.org/organisms/campylobacter-non-
71 jejunicoli/c-upsaliensis-primers). DNA sequences of the seven genes used for the MLST

72 analysis were assembled using BioNumerics 7.6 software (Applied Maths, Belgium). Novel
73 alleles and sequence types (STs) were assigned following submission of the DNA sequences
74 and profiles to the non *jejuni/coli Campylobacter* PubMLST database
75 (http://pubmlst.org/campylobacter/).
76 Concatenated sequences representing all *C. upsaliensis* sequence types within the
77 *Campylobacter* PubMLST database were downloaded from PubMLST and combined with
78 sequences representing the profiles from this study. A concatenated sequence based on the *C.*
79 *upsaliensis* MLST gene get was also extracted from the type strain genome of *C. helveticus.*
80 These sequences were imported into BioNumerics and aligned using the Fast algorithm.
81 Within BioNumerics, a Neighbor-joining dendrogram was constructed from the aligned
82 profile sequences. A minimum spanning tree (MST) was constructed based on the sequence
83 distances between the concatenated profile sequences and using the default priority rules,
84 'Permutation resampling' resampling strategy and 'Highscore summary' methods. MST
85 nodes were color-coded within BioNumerics according to taxon.
86
87 *Biochemical and microscopic characterization*
88 The 14 isolates were tested for the following phenotypic traits: oxidase, catalase, urease and
89 alkaline phosphatase activity; hydrolysis of hippurate and indoxyl acetate; reduction of
90 nitrate, selenite and 2,3,5-triphenyltetrazolium chloride (TTC); H_2S production on triple
91 sugar iron agar; α-haemolysis; growth at 25 °C, 30 °C, 37 °C and 42 °C; growth under
92 aerobic, microaerobic and anaerobic conditions; growth on CCDA and on media amended
93 with 1% (w/v) glycine, 2% (w/v) NaCl or 1% (w/v) bile. Resistance to nalidixic acid and
94 cephalothin was also determined. All tests were performed using standard methods as
95 previously described [35-39] and were conducted at least twice.

96 Morphological characteristics of three of the novel strains were determined using
97 transmission electron microscopy. Cells grown on Nutrient Broth No.2 (Oxoid) with 1.5% of
98 Bacto Agar (Difco) and 10% of sheep blood for 48 h were gently suspended in 0.1 M
99 phosphate-buffered saline (PBS) at a concentration of about 108 cells per ml. Samples were
100 negatively stained with 2% (w/v) phosphotungstic acid (pH 6.5) for 30 s. The specimens were
101 then examined using a Philips EM208S TEM.
102
103 *MALDI-TOF MS*
104 A Microflex LT/SH MALDI-TOF MS (Bruker Daltonics, Germany) with a 60-Hz nitrogen
105 laser was used to analyze spectra over a mass range of 2,000-20,000 Da. All the isolates and
106 *C. upsaliensis* ATCC 43954^T were processed according to the manufacturer's instructions. A
107 sterile wooden tip was used to pick an isolated bacterial colony freshly grown on Mueller-
108 Hinton agar (Oxoid) supplemented with 5% sheep blood for 48 h at 37 °C under
109 microaerobic conditions. A thin film in five replicates was then smeared onto a MALDI 96-
110 target polished steel plate (Direct Transfer procedure). Microbial films were overlaid directly
111 with 1.0 µl of a 10 mg/ml α-cyano-4-hydroxycinnamic acid (MALDI-TOF HCCA) matrix
112 solution. The sample-matrix mixture was dried at room temperature and subsequently
113 inserted into the system for data acquisition. The spectra were constructed based on processed
114 raw spectra after smoothing, baseline subtraction, normalization, and peak picking. For each
115 spot, 50 sub-spectra for each of 40 randomized positions within the spot were collected and
116 presented as one main spectrum. A sum spectrum was acquired by summing the laser shots.
117 Quality controls were internally calibrated using the *Escherichia coli* DH5α supplied by
118 Bruker Daltonics, following the same procedure. The data were processed automatically by
119 the instrument software, and the spectra were compared with reference libraries for bacterial
120 identification matching. Spectra were analyzed using MBT Compass 4.1.70.1 database

121 version 7.0.0.0 (Bruker Daltonics, Germany). Manufacturer-recommended score cut-offs
122 were used to determine genus level (1.7000 to 1.999) or species level ($\geq$2.000) of the
123 organism. A score of <1.7 was considered unreliable for genus identification. Further
124 analyses were performed using the ClinProTools 3.0 software package (Bruker Daltonics,
125 Germany). For all the characteristic proteins, the results of the following statistical tests were
126 taken into consideration: Anderson-Darling, Wilcoxon, t-test, Variation Coefficient and ROC
127 curves.

128

129 *rDNA,* atpA *and core gene phylogenetic analysis*

130 The 16S rDNA and *atpA* genes were amplified by the universal internal primers p27f and
131 p1492r [7] and atpAFC3 and atpARC4 [33], respectively, and then sequenced with an ABI
132 3730 DNA analyzer at StarSEQ GmbH (Mainz, Germany). For each gene set, the sequences
133 from the 14 strains were combined with gene sequences extracted from the *Campylobacter*
134 type strain genomes (defined hereafter as the genomes of all validly-described
135 *Campylobacter* species type strains, the genome of *C. portucalensis* sp. nov. strain LMG
136 31504^T [46], and the genomes of *C. aviculae* sp. nov. strain LMG 31272^T, *C. estrildidarum*
137 sp. nov. strain LMG 31271^T and *C. taeniopygiae* sp. nov. strain LMG 30935^T [8]) and the
138 *Helicobacter pylori* type strain genome (as an outgroup) and aligned using Clustal X [25].
139 Neighbor-Joining trees were generated from these alignments in MEGA6 [48], using the
140 Kimura 2-parameter method.

141 Additionally, the sequences of 20 core genes (*aroC*, *atpA*, *dnaN*, *eno*, *fabH*, *frr*, *glnA*, *groEL*,
142 *hemB*, *ileS*, *lpxA*, *miaB*, *mrp*, *nrdB*, *pnp*, *prfA*, *queA*, *speA*, *spoT* and *tkt*) were extracted from
143 the genomes of the 14 strains, the *Campylobacter* type strain genomes, and the *Helicobacter*
144 *pylori* type strain genome (as an outgroup). The genes were aligned individually using
145 MUSCLE in Geneious Prime (v. 2020.0.5) with default parameters. The 20 alignments were

146 then concatenated alphabetically and a Neighbor-Joining dendrogram was constructed in
147 Geneious Prime using the Tamura-Nei genetic distance model.
148
149 *Whole genome sequencing and annotation*
150 The complete genome sequence of the type strain 251/13^T was determined using PacBio
151 sequencing; draft genome sequences of the remaining strains were obtained using Illumina
152 sequencing. Illumina sequencing was also performed on the type strain to improve PacBio
153 base calling. For all 14 strains, genomic DNAs were prepared from loopfuls of cells using the
154 Promega Wizard Genomic DNA Purification Kit. The same genomic DNA preparation was
155 used to construct the Illumina and PacBio libraries for the type strain. Illumina libraries were
156 prepared using the Illumina Nextera DNA Flex Library Prep Kit with 20ng of genomic DNA.
157 Pooled libraries were sequenced on a MiSeq instrument at 8.0 pM, with dual index reads,
158 paired end, using the MiSeq Reagent Kit v2 (300-cycles). For the type strain, a 20 kb PacBio
159 library was prepared using 15 µg of genomic DNA, the PacBio SMRTbell Template Prep Kit
160 1.0, and manufacturer's protocols. SMRT sequencing was performed on an RSII sequencer.
161 PacBio reads were assembled using the Hierarchical Genome Assembly Process (HGAP ver.
162 3.0) in the SMRT Analysis software (ver. 2.3.0). Sequencing metrics for all 14 strains are
163 presented in Supplementary Table 1. PacBio sequencing of strain 251/13^T resulted in two
164 contigs: one representing the chromosome and another representing a 36,943 bp plasmid,
165 termed pVULP. Both contigs were circularized manually within Geneious Prime (ver.
166 2019.1.3; Biomatters Ltd., Auckland, New Zealand), and base calling was further improved
167 by mapping the MiSeq reads onto the circularized PacBio contigs within Geneious Prime.
168 Using the Geneious "Find Variations/SNPs" module, with a default minimum variation of
169 0.3, genomic variations in each contig were identified and corrected to the MiSeq consensus
170 sequence.

171 Annotation of the type strain genome was performed manually. Putative coding sequences,
172 ribosomal loci and tRNA/transfer-messenger RNA (tmRNA) genes were identified using
173 GeneMark, RNAmmer and ARAGORN [3, 24, 26] respectively. The start point of each
174 coding sequence was curated manually within Artemis (v.16) [44]. Gene function was
175 initially assigned following a BLASTP comparison of the 251/13^T proteome against the
176 proteomes derived from the other annotated *Campylobacter* type strains. Annotation was
177 further refined by identification of Pfam motifs [13] and a BLASTP analysis that utilized the
178 larger NCBI non-redundant (nr) protein database. Annotation of the thirteen draft genomes
179 was performed by means of the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [49]
180 upon submission of the genomes to GenBank.
181
182 *Whole-genome analyses*
183 Analyses based on average nucleotide and amino acid identities and *in silico* DNA-DNA
184 hybridization were used to determine if the new isolates belonged to any of the previously-
185 characterized species of *Campylobacter*. The average nucleotide identity based on BLAST
186 (ANIb) analysis was performed using JSpecies (v. 1.2.1) [42]. The data set analyzed included
187 the complete 251/13^T genome and the *Campylobacter* type strain genomes. The proteomes of
188 the same taxa used in the ANI analysis were compiled into a single FASTA-formatted 72,437
189 protein amino acid sequence file. An all-against-all BLASTP [2] analysis was performed
190 using this composite proteome file. The BLASTP output was subsequently analyzed with
191 BlastPTrimmer14 [34] using 35% sequence similarity and 75% alignment length as input
192 parameters, which identified the proteins conserved among the 43 taxa and generated pair-
193 wise average amino-acid identity (AAI) values using the core protein set. In silico DNA-
194 DNA hybridization (isDDH) values were determined using the Genome-to-Genome Distance
195 Calculator 2.1 (GGDC; https://ggdc.dsmz.de/ggdc.php#; accessed Sept 2020) [30]. ANI, AAI

196 and isDDH analyses were also performed on a 28-strain set that included an additional 25 *C.*
197 *vulpis*, *C. upsaliensis* and *C. helveticus* strains. In addition to the three complete *C. vulpis*, *C.*
198 *upsaliensis* and *C. helveticus* type strain genomes and the thirteen *C. vulpis* draft genomes
199 sequenced here, ten *C. upsaliensis* genomes and two *C. helveticus* genomes were downloaded
200 from NCBI. ANIb, AAI and isDDH analyses were performed as described above, using a
201 FASTA-formatted 48,744 protein amino acid sequence file for the AAI analysis. Whole-
202 genome based average nucleotide identity (gANI) analysis was performed using the JGI
203 Microbial Species Identifier (MiSI; https://ani.jgi.doe.gov/html/home.php?; accessed Mar
204 2021 [51]) and FASTA-formatted coding sequence files. The second isDDH analysis used a
205 mixture of draft and complete genomes; therefore, Formula 2 (recommended for draft
206 genomes as it is independent of genome length) was used throughout both isDDH analyses to
207 maintain consistency.
208
209 *Clusters of Orthologous Genes (COGS) and Rarefaction Analyses*
210 Clusters of reciprocal best BLASTP matches [putative Clusters of Orthologous Genes
211 (COGs)] were established using all-against-all BLASTP searches, which employed the
212 BLOSUM80 matrix and accepted only the best reciprocal matches with E-values $\leq 1\times10^{-5}$,
213 where "second-best" matches produced bit scores < 90% of those associated with the best
214 matches, in order to avoid confounding effects deriving from recent duplications and
215 paralogous genes. The estimated size of the core and accessory genome, based on the COGs
216 identified by the method described above, were recorded for all possible combinations of the
217 2 to 14 genomes included in this study. The core genome of the genus *Campylobacter* was
218 determined by applying the same method to a collection of 38 *Campylobacter* type strain
219 proteomes, with the inclusion of 251/13^T as the type strain of the putative new species. Plots
220 were prepared showing mean and standard deviation of these combinations. The scripts used

221 for the identification of COGs and execution of rarefaction analysis for the estimation of the
222 size of the core and accessory genomes of the studied isolates are available at
223 https://github.com/cvulpispaper/compute_aai_and_cogs.
224
225 *Identification of "plasmid-like" sequences*
226 The complete collection of plasmid sequences included in the PLSDB database [16] was
227 retrieved from https://ccb-microbe.cs.uni-saarland.de/plsdb/plasmids/download/?zip
228 (accessed Sept 2020). Sequence similarity searches against the PLSDB were performed using
229 BLASTN, as available from the BLAST+ suite [10].
230
231 *Species-specific PCR*
232 In order to define a diagnostic method for the rapid detection and identification of the
233 putative new species, specific PCR primers targeting the *atpA* gene were designed:
234 atp_FW18 5'-TGC CGC TTT ACA ATA TCT CGC T-3' and atp_Rev405 5'-CCC CAC
235 ACG CGA AAC AGA CAA G-3'. Amplification parameters were as follows: 30 cycles of
236 30 s at 94 °C, 30 s at 58 °C and 60 s at 72 °C, preceded by a denaturation step at 95 °C for 5
237 min and followed by an extended elongation step at 72 °C for 7 min. To assess the sensitivity
238 and specificity of this PCR method, all 14 strains and a selection of strains representing
239 different species of *Campylobacter* were analyzed using the specified conditions.
240
241 *Data availability*
242 The GenBank accession numbers for the 16S rRNA gene and *atpA* sequences of strains
243 36/13, 43/13, 52/13, 73/13, 99/13, 108/13, 131/13, 146/13, 147/13, 154/13, 166/13, 227/13,
244 250/13 and 251/13^T are KU855032-KU855045 (16S) and KU855018-KU855031 (*atpA*). The
245 complete sequence of the type strain 251/13^T has been deposited in GenBank [CP041617

246 (chromosome) and CP041618 (megaplasmid pVULP)]. The draft genome sequences of the
247 other strains have been deposited in GenBank with the following accession numbers:
248 VJYO00000000 (36/13), VJYW00000000 (43/13), VJYU00000000 (52/13),
249 VJYY00000000 (73/13), VJYM00000000 (99/13), VJYT00000000 (108/13),
250 VJYV00000000 (131/13), VJYN00000000 (146/13), VJYS00000000 (147/13),
251 VJYX00000000 (154/13), VJYQ00000000 (166/13), VJYP00000000 (227/13),
252 VJYR00000000 (250/13).

253

254 **RESULTS AND DISCUSSION**

255 *Morphology and phenotypic characterization*

256 After 48 h of incubation at 37 ± 1°C, colonies appear 2-3 mm in diameter, α-haemolytic,
257 grey, translucent, flat with an irregular edge, and show a tendency to spread along the
258 direction of the streak and to swarm and coalesce. Cells are Gram-negative, sigmoid to
259 allantoid in shape, 0.3-0.4 µm in width and 1.2-3.0 µm in length with one bipolar unsheathed
260 flagellum (Fig. 1), and motile with characteristic darting movements when observed by dark
261 field microscopy. Cells appear coccoid after 5-6 days of incubation or when exposed to air.
262 All 14 isolates from red foxes were identified initially as *C. upsaliensis* according to the
263 results of a *Campylobacter* genus-specific PCR and a species-specific PCR for *C. upsaliensis*
264 and *C. helveticus*. Thus, it was not unexpected that results of the standard biochemical and
265 growth tests showed a strong similarity between the composite phenotypic profile observed
266 for the 14 fox isolates and the phenotypic profile reported previously for *C. upsaliensis*
267 (Table 1). Nevertheless, the 14 fox isolates could be unambiguously distinguished from *C.*
268 *upsaliensis* by their inability to grow at 30 °C under microaerobic conditions (Table 1).
269 Additionally, alkaline phosphatase activity is variable within our strain set but negative in *C.*
270 *upsaliensis*, and TTC reduction is negative in our strain set but variable in *C. upsaliensis*.

271 Although the profile from our strain set is also highly similar to the profile reported for *C.*
272 *helveticus*, they are clearly distinguished from *C. helveticus* by their ability to reduce selenite
273 (Table 1).
274
275 *MALDI-TOF analysis*
276 We determined if our 14 fox isolates could also be distinguished from *C. upsaliensis* using
277 MALDI-TOF MS. MALDI-TOF MS has been used in previous descriptions of novel
278 *Campylobacter* taxa; *Campylobacter fetus* subsp. *testudinum* could not be distinguished from
279 *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* by standard phenotypic tests but were
280 clearly distinct from those subspecies based on MALDI-TOF MS profiles [15], and MALDI-
281 TOF MS analysis was used as additional supporting evidence in the description of
282 *Campylobacter armoricus* [6].
283 MALDI-TOF MS spectra were generated for the 14 strains. The result "*No Organism*
284 *Identification Possible*" was obtained by comparing these experimental mass spectra against
285 the data contained in the Compass database used in this study. The average mass spectra of
286 all acquired experimental data for *C. upsaliensis* NCTC 11541^T and the 14 isolates overlap
287 across the entire mass range, although some differences are noticeable in the protein profiles.
288 In the spectral regions between *m/z* 8200-8600 and *m/z* 10180-10260, there are characteristic
289 peaks for both species (Supplementary Fig. 1). Specific peaks at *m/z* values of 8376, 8424
290 and 10201 for *C. upsaliensis* NCTC 11541^T and *m/z* values of 8510 and 10227 for two fox
291 strains (251/13^T; 73/13), as monoprotonated molecules, were identified (Supplementary Fig.
292 1). The same proteins are revealed in the mass spectra as doubly-charged ions, as
293 demonstrated by the replication of the same profiles in the spectral range between *m/z* 4180-
294 4270 and 5095-5120 (Supplementary Fig. 1). The results of the Variation Coefficient tests, in
295 terms of standard deviation of intensity values, and the ROC curves, in terms of Area Under

296 the Curve (AUC), reveal a statistical significance for the peaks under study. Finally, main
297 spectra library (MSP) phylogenetic analysis based on the average mass spectra of all acquired
298 experimental data for the fox isolates formed a clade distinct from the other *Campylobacter*
299 taxa present in the MBT Compass software database (Supplementary Fig. 2); this clade is
300 also clearly distinct from the clade containing *C. upsaliensis*. Thus, the proteomic data
301 indicate that the isolates under study, hereafter named *C. vulpis*, exhibit a specific protein
302 pattern that could also be used to discriminate them from the closely-related *C. upsaliensis*.

303

304 C. vulpis *multi-locus sequence typing*

305 MLST was performed on the 14 *C. vulpis* strains. For each of the seven MLST genes and for
306 all 14 isolates, the MLST alleles identified here are notably different, when compared to the
307 *C. upsaliensis* alleles present in the *Campylobacter* MLST database, and all the identified
308 alleles and STs are novel. Nine sequence types were identified among the 14 *C. vulpis* strains,
309 indicating genotypic diversity within the strain set. A minimum spanning tree was created
310 from the MLST profile sequences of *C. vulpis*, the type strain of *C. helveticus*, and all *C.*
311 *upsaliensis* sequence types currently present within PubMLST. Within this tree, the *C. vulpis*
312 isolates group together and are clearly separated from *C. upsaliensis* (Fig. 2).

313

314 *16S rDNA,* atpA *and core gene phylogenetic analysis*

315 Alignment of the 16S rDNA sequences from *C. vulpis* and the *C. upsaliensis* type strain
316 indicated identity (0% sequence distance) between the *C. vulpis* strains, with near identity
317 (99.8% similarity) to the 16S rDNA sequence of the *C. upsaliensis* type strain. This is
318 reflected in the dendrogram constructed from the aligned sequences (Fig. 3). However,
319 similarity values between the *C. vulpis* 16S rDNA loci and those of five additional *C.*

320 *upsaliensis* strains range from 98.5% to 99.8% (data not shown). High similarity between the
321 16S rDNA loci of related taxa has been reported previously in *Campylobacter*; the 16S rDNA
322 sequences of three novel species recovered from Zebra finches are 99% similar [8].
323 Nevertheless, the neighbor-joining tree demonstrates that our strains and the *C. upsaliensis*
324 type strain form a well-supported (92% bootstrap) clade that is separate from the rest of the
325 *Campylobacter* species included in the dataset (Fig. 3).

326 To further investigate the taxonomic position of *C. vulpis,* we used *atpA* sequencing, which
327 has been shown to have a higher resolving power than 16S-based analyses in *Campylobacter*
328 [33].

329 In this analysis, the *C. vulpis* strains form a clade that is clearly distinct from the other
330 *Campylobacter* taxa, including *C. upsaliensis* (Fig. 4). A phylogenetic tree was also
331 constructed using the concatenated sequences of 20 core genes (Supplementary Fig. 3).
332 Consistent with the results of the *atpA* analysis, *C. vulpis* forms a clade that is distinct from
333 the other *Campylobacter* taxa.

334

335 *Whole genome sequencing and analysis*

336 The genome of the *C. vulpis* type strain 251/13 was sequenced to completion using a
337 combination of PacBio and Illumina MiSeq sequencing. The genome of $251/13^T$ is 1,645 kbp
338 with a G+C content of 34.7% (Supplementary Table 1). The $251/13^T$ genome putatively
339 encodes 1564 genes, 52 pseudogenes and 3 ribosomal loci, although 2 of these ribosomal loci
340 are split, as observed also in the related species *C. helveticus* [32]. The $251/13^T$ genome
341 contains three genomic islands (43, 33, and 19 kbp) and no CRISPR/Cas loci. A 36,943 bp
342 plasmid, termed pVULP, was also identified in the $251/13^T$ genome; this plasmid is
343 presumably conjugative, due to the presence of genes encoding a P-type type IV conjugative
344 transfer system. The other thirteen genomes were sequenced to draft level by Illumina MiSeq

345 sequencing (Supplementary Table 1). The approximate sizes of these genomes range from
346 1,574 to 1,724 kbp with G+C contents ranging from 34.4 to 34.7%. These %G+C values are
347 similar to those reported for *C. upsaliensis* (i.e., 34-35%) and are within the range reported
348 for other members of the genus *Campylobacter* (29–47 %) [12]. BLASTN analysis of the *C.*
349 *vulpis* draft genomes, using pVULP as a query sequence, indicated the potential presence of
350 plasmids in nine of the thirteen strains (Supplementary Table 1); these results were confirmed
351 by BLAST sequence similarity searches against the PLSDB database, a specialized database
352 containing the complete sequences of more than 18,000 plasmids. However, it is possible that
353 these plasmid sequences in the draft genomes are located in genomic islands rather than on
354 extrachromosomal elements; in addition to pVULP, P-type type IV conjugative transfer
355 system encoding genes are also located within the 19 and 33 kbp genomic islands on the
356 251/13^{T} chromosome. Therefore, determination of the plasmid content of the draft genomes
357 may require genome closure.
358 Rarefaction analysis based on the *C. vulpis* genomes sequenced in this study (Fig. 5),
359 indicates that the core genome of *C. vulpis* is 1176 genes and accounts for more than 70% of
360 the average *C. vulpis* gene content. Furthermore, the core genome curve illustrated in Figure
361 5 suggests that the size of the core genome is not likely to be affected substantially by the
362 sequencing of additional genomes. However, the accessory genome presently contains 1260
363 genes. This is clearly a minimum value (Fig. 5), suggesting that currently available data offer
364 only a partial representation of the pan-genome of *C. vulpis*.
365
366 *Whole genome comparisons*
367 Average nucleotide identity based on BLAST (ANIb) analysis indicated that the *C. vulpis*
368 type strain showed the highest levels of sequence identity with the *C. upsaliensis* type strain,
369 with an ANIb value of 89.9 (Supplementary Table 2A). Similar values were observed for the

370 other 13 strains, when compared to the *C. upsaliensis* type strain, with an average ANIb value
371 of 90.1% (Table 2, Supplementary Table 2B). The average ANIb value between *C. vulpis*
372 strains was 99.2% (Supplementary Table 2B). Importantly, ANIb values determined for every
373 isolate considered in this study are well below the 94-95% cut-off that has been recently
374 recommended for the discrimination of bacterial species [18, 23, 42]. Moreover, the average
375 pairwise *C. vulpis/C. upsaliensis*^{T} ANIb value is below the ANIb values observed for all
376 currently-recognized *Campylobacter* subspecies pairs: *C. jejuni* subspp. *jejuni* and *doylei*
377 (96.1%); *C. lari* subspp. *lari* and *concheus* (93.2%); *C. fetus* subspp. *fetus/venerealis* and
378 *testudinum* (91.8%); *C. hyointestinalis* subspp. *hyointestinalis* and *lawsonii* (94.2%); and *C.*
379 *pinnipediorum* subspp. *caledonicus* and *pinnipediorum* (94.3%) (Supplementary Table 2A).
380 This average pairwise ANIb value is also below that observed between *C. lari* subsp.
381 *concheus* and *C. ornithocola* (91.0%; Supplementary Table 2A).
382 To further characterize the average nucleotide identities within *C. vulpis, C. upsaliensis* and
383 *C. helveticus*, we used whole-genome based average nucleotide identity (gANI) analysis in
384 addition to ANIb on an expanded strain set that included all 14 *C. vulpis* strains, 11 *C.*
385 *upsaliensis* strains and 3 *C. helveticus* strains. Each method calculates the ANI in a different
386 fashion: ANIb cuts the query genome into 1020 nt fragments which are then used to search
387 against the reference genome using BLASTN [18], whereas gANI utilizes FASTA-formatted
388 coding sequence files for each genome, which are then compared using BLASTN [23].
389 Although, in general, the gANI value was slightly higher than the ANIb value for each strain
390 pair, the two ANI methods yielded very similar results (Supplementary Table 2B).
391 Interestingly, ANI analysis within *C. upsaliensis* identified two strains, RM3195 and NCTC
392 12264, that are more divergent than the other nine strains, with intergroup ANI values of
393 ~96.6% (Supplementary Table 2B). Nevertheless, despite this variation within *C. upsaliensis*,
394 both ANI methods yielded *C. vulpis* vs. *C. upsaliensis* values [90.1 ± 0.2 (ANIb); 90.4 ± 0.2

395 (gANI)] well below the 94-95% ANI species boundary (Supplementary Table 2B). Palmer et
396 al. [40] concluded that the various described ANI methods do not completely correlate within
397 the region of 90-100% similarity, a region that includes the proposed ANI species boundary.
398 This lack of correlation would be more critical if the calculated ANI values were in close
399 proximity to the species boundary, i.e. ~94-95%. However, the *C. vulpis* vs. *C. upsaliensis*
400 ANI values determined here, which are ~4% below the species boundary using either ANI
401 method, strongly suggest that the *C. vulpis* and *C. upsaliensis* strains comprise two distinct
402 *Campylobacter* species, a conclusion that is likely ANI method independent.
403 Average amino acid identity (AAI) analysis, based on the determination of pairwise
404 similarities among the *Campylobacter* core protein set, indicated that the *C. vulpis* core
405 proteins are most similar to those of the *C. upsaliensis* type strain, with an average AAI of
406 95% (Table 2). *Campylobacter vulpis* core proteins are also similar to those of *C. helveticus*
407 (AAI = 91%; Table 2). AAI values between *C. vulpis* and the remaining *Campylobacter* taxa
408 were ≤ 81% (Table 2, Supplementary Table 2A). AAI analysis using the expanded strain set
409 described above yielded similar results. The average pairwise AAI value between the 14 *C.*
410 *vulpis* strains and the 11 *C. upsaliensis* strains was 94.6% (Supplementary Table 2C), which
411 is below the intraspecific *C. vulpis* and *C. upsaliensis* values of 99.6% and 98.8%,
412 respectively (Supplementary Table 2C). Here also, AAI values between *C. vulpis* and *C.*
413 *upsaliensis* are below those observed within established *Campylobacter* subspecies pairs
414 (Supplementary Table 2A). Additionally, the AAI values are similar to those observed
415 between some *Campylobacter* species [e.g., *C. rectus* and *C. showae* (95%), and *C.*
416 *subantarcticus* and *C. lari* (95%); Supplementary Table 2A].
417 The estimated in silico DNA-DNA hybridization (isDDH) value between the *C. upsaliensis*
418 type strain genome and the *C. vulpis* type strain genome is 40.1% (CI [37.7 – 42.7%])
419 (Supplementary Table 3). Analysis of the expanded strain set yielded a similar average value

420 (40.2%; Supplementary Table 2C). isDDH analysis using the 28-strain set is complicated by
421 the mixture of draft and complete genomes. Formulas 2 and 3 on the GGDC service divide
422 the high-scoring segment pairs (HSPs) by HSP length or genome length, respectively. Thus,
423 Formula 2 is recommended by the GGDC service for draft genomes of uncertain genome
424 length, and we utilized Formula 2 throughout this study to maintain consistency. Notably, the
425 variation within *C. upsaliensis* described above is also observed here, with isDDH values of
426 ~70% when strains RM3195 and NCTC 12264 were compared to the other nine *C.*
427 *upsaliensis* strains (Supplementary Table 2C). However, as with the ANI results, this
428 variation within *C. upsaliensis* did not substantially affect the pairwise *C. vulpis/C.*
429 *upsaliensis* isDDH values, although these data may indicate that further research on *C.*
430 *upsaliensis* taxonomy may be warranted. Although these isDDH data support the description
431 of *C. vulpis* as a novel *Campylobacter* species, the presence of incomplete genomes within
432 the data set suggest that isDDH analysis should not be used as the sole taxonomic
433 determinant.
434
435 C. vulpis-*specific PCR*
436 To specifically amplify *C. vulpis* strains, a novel primer pair was designed that targets that
437 *atpA* gene. Amplification of all 14 *C. vulpis* isolates produced a 409 bp PCR product,
438 whereas the other *Campylobacter* species were PCR-negative. Additionally, the 39 *C.*
439 *upsaliensis* strains characterized by Rossi et al. [43] from domestic dogs and cats were also
440 PCR-negative. DNA sequencing of the PCR products from *C. vulpis* confirmed the
441 specificity of the amplicons. This suggests that the *C. vulpis*-specific PCR is robust and can
442 be used to rapidly discriminate between *C. vulpis* and *C. upsaliensis* strains.
443
444 ***CONCLUSIONS***

445 Fourteen *Campylobacter* isolates were recovered from the cecal contents of wild red foxes in
446 Northern Italy in 2013. These strains were identified initially as *C. upsaliensis* by
447 *Campylobacter* species-specific PCR; however, MLST placed these isolates in a clade
448 separate from *C. upsaliensis.* MLST also identified nine sequence types within the 14
449 isolates, indicating genomic variation within the strain set. Although phenotypically similar
450 to both *C. upsaliensis* and *C. helveticus,* these 14 fox isolates could be distinguished from *C.*
451 *upsaliensis* and *C. helveticus* by microaerobic growth at 30 °C and reduction of selenite,
452 respectively. Discrimination of the fox isolates and *C. upsaliensis* could also be observed
453 using MALDI-TOF MS analysis. These results suggested that the 14 fox isolates, although
454 related to *C. upsaliensis* and *C. helveticus,* were representatives of a novel species distinct
455 from other validly-described species of the genus *Campylobacter*. The name *Campylobacter*
456 *vulpis* sp. nov. was proposed, with isolate 251/13^T (=CCUG 70587^T; =LMG 30110^T) as the
457 type strain. 16S rDNA, *atp*A, core gene phylogenetic analyses and whole-genome sequence
458 comparisons, including ANI, AAI and isDDH analyses, were also performed to further
459 address the taxonomic placement of *C. vulpis*. *atpA* and core gene dendrograms placed *C.*
460 *vulpis* strains into clades related to but well separated from *C. upsaliensis.* Additionally,
461 pairwise *C. vulpis/C. upsaliensis* ANI and AAI values were below those observed for existing
462 *Campylobacter* subspecies pairs but consistent with other *Campylobacter* species pairs.
463 Taken together, these data support the designation of *C. vulpis* sp. nov. as a novel
464 *Campylobacter* species. A robust *C. vulpis*–specific PCR was developed which will permit
465 the rapid identification of *C. vulpis* strains recovered from animal, environmental, or possibly
466 human clinical samples. Description of *Campylobacter vulpis* sp. nov. is presented in Table
467 3.
468
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480
481 **DECLARATION OF COMPETING INTEREST**
482 The authors declare that they have no conflicts of interest.
483
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639

640 **FIGURE AND SUPPLEMENTARY FIGURE LEGENDS**

641

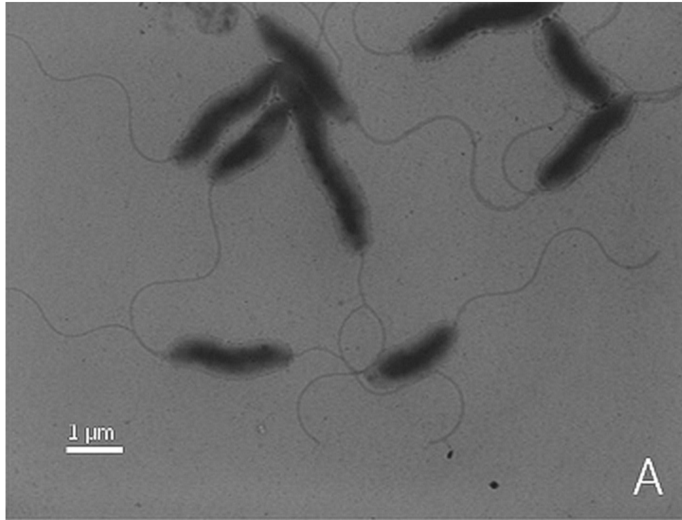
642 **Figure 1.** Electron micrograph of *C. vulpis* strain 251/13^T cells from a 48 h culture. (a) Cells
643 with bipolar flagella. (b) Detail of unsheathed flagellum.
644
645 **Figure 2.** MLST-based minimum spanning tree illustrating the genotypic relationships of the
646 *C. vulpis* sp. nov. strains from this study and strains representing all *C. upsaliensis* sequence
647 types present within the PubMLST database (https://pubmlst.org/campylobacter/; accessed
648 Sept 2020). The *C. helveticus* MLST locus set is different from that of *C. upsaliensis*; thus, to
649 ensure consistency, allele sequences from the locus set used in the *C. upsaliensis* MLST
650 method were extracted from the *C. helveticus* type strain genome sequence. Nodes derived
651 from the type strains are labeled with a 'T'. *C. vulpis* nodes are labeled with their respective
652 strains.
653
654 **Figure 3.** 16S phylogenetic tree representing *Campylobacter vulpis* sp. nov. and the
655 *Campylobacter* type strains. The evolutionary history was inferred using the Neighbor-
656 Joining method [45]. The percentage (>75%) of replicate trees in which the associated taxa
657 clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The
658 tree is drawn to scale, with branch lengths in the same units as those of the evolutionary
659 distances used to infer the phylogenetic tree. The evolutionary distances were computed
660 using the Kimura 2-parameter method [22] and are in the units of the number of base
661 substitutions per site.
662
663 **Figure 4.** *atpA* phylogenetic tree representing *Campylobacter vulpis* sp. nov. and the
664 *Campylobacter* type strains. The evolutionary history was inferred using the Neighbor-
665 Joining method [45]. The percentage (>75%) of replicate trees in which the associated taxa
666 clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The

667 tree is drawn to scale, with branch lengths in the same units as those of the evolutionary
668 distances used to infer the phylogenetic tree. The evolutionary distances were computed
669 using the Kimura 2-parameter method [22] and are in the units of the number of base
670 substitutions per site.

671

672 **Figure 5.** Core and accessory genome of *C. vulpis* sp. nov. Core genome, blue. Accessory
673 genome, orange. The number of isolates considered is indicated on the x axis, while the
674 number of genes is represented on the y axis. Error bars are used to indicate the standard
675 deviation.

676



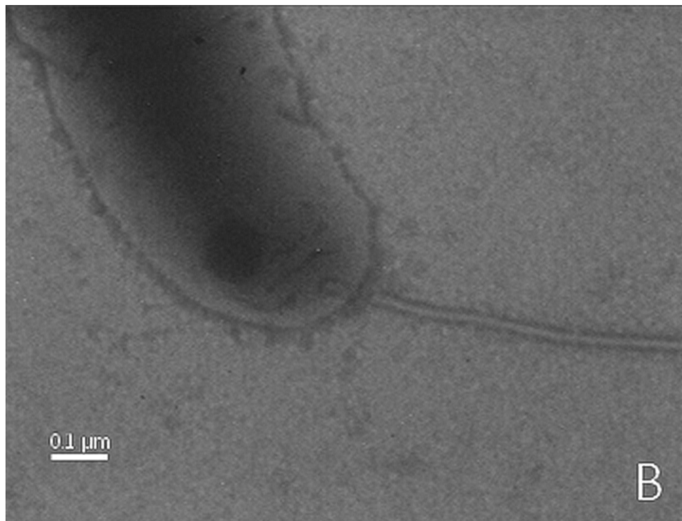


Figure 1. Electron micrograph of *C. vulpis* strain 251/13^{T} cells from a 48 h culture. (a) Cells with bipolar flagella. (b) Detail of unsheathed flagellum.

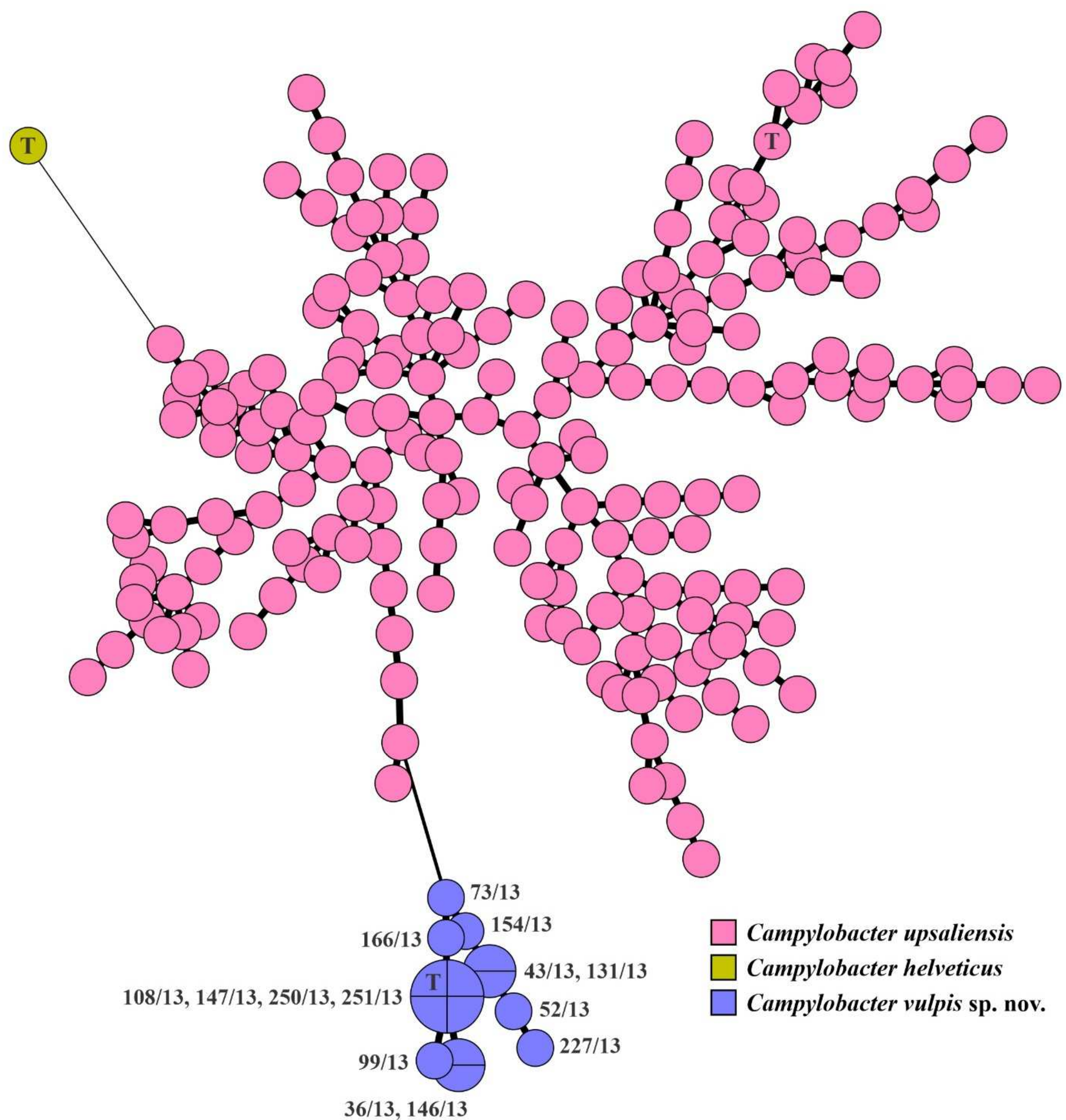


Figure 2. MLST-based minimum spanning tree illustrating the genotypic relationships of the *C. vulpis* sp. nov. strains from this study and strains representing all *C. upsaliensis* sequence types present within the PubMLST database (https://pubmlst.org/campylobacter/; accessed 9/14/2020). The *C. helveticus* MLST locus set is different from that of *C. upsaliensis*; thus, to ensure consistency, allele sequences from the locus set used in the *C. upsaliensis* MLST method were extracted from the *C. helveticus* type strain genome sequence. Nodes derived from the type strains are labeled with a 'T'. *C. vulpis* nodes are labeled with their respective strains.

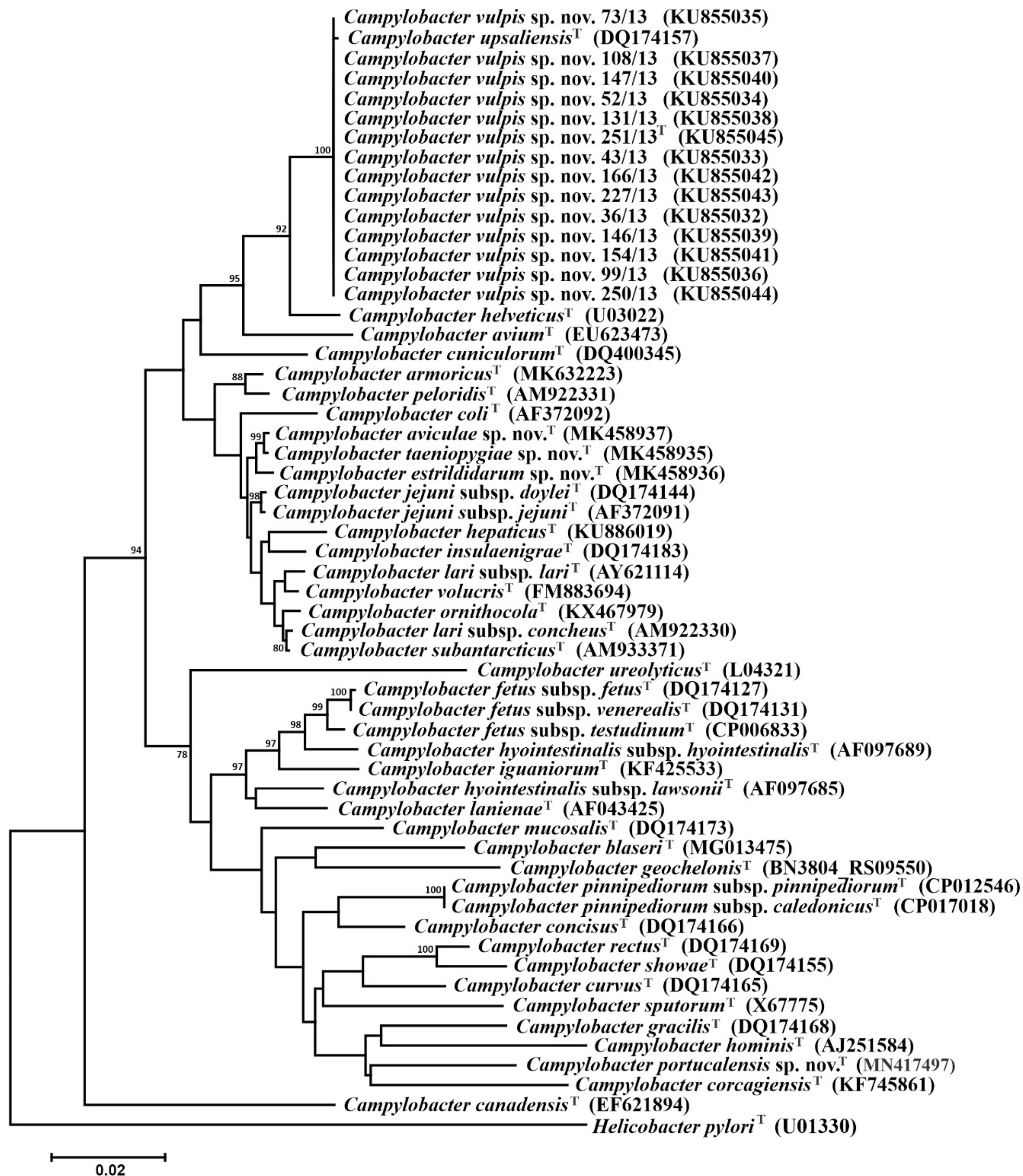


Figure 3: 16S phylogenetic tree representing *Campylobacter vulpis* sp. nov. and the *Campylobacter* type strains. The evolutionary history was inferred using the Neighbor-Joining method [45]. The percentage (>75%) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [22] and are in the units of the number of base substitutions per site.

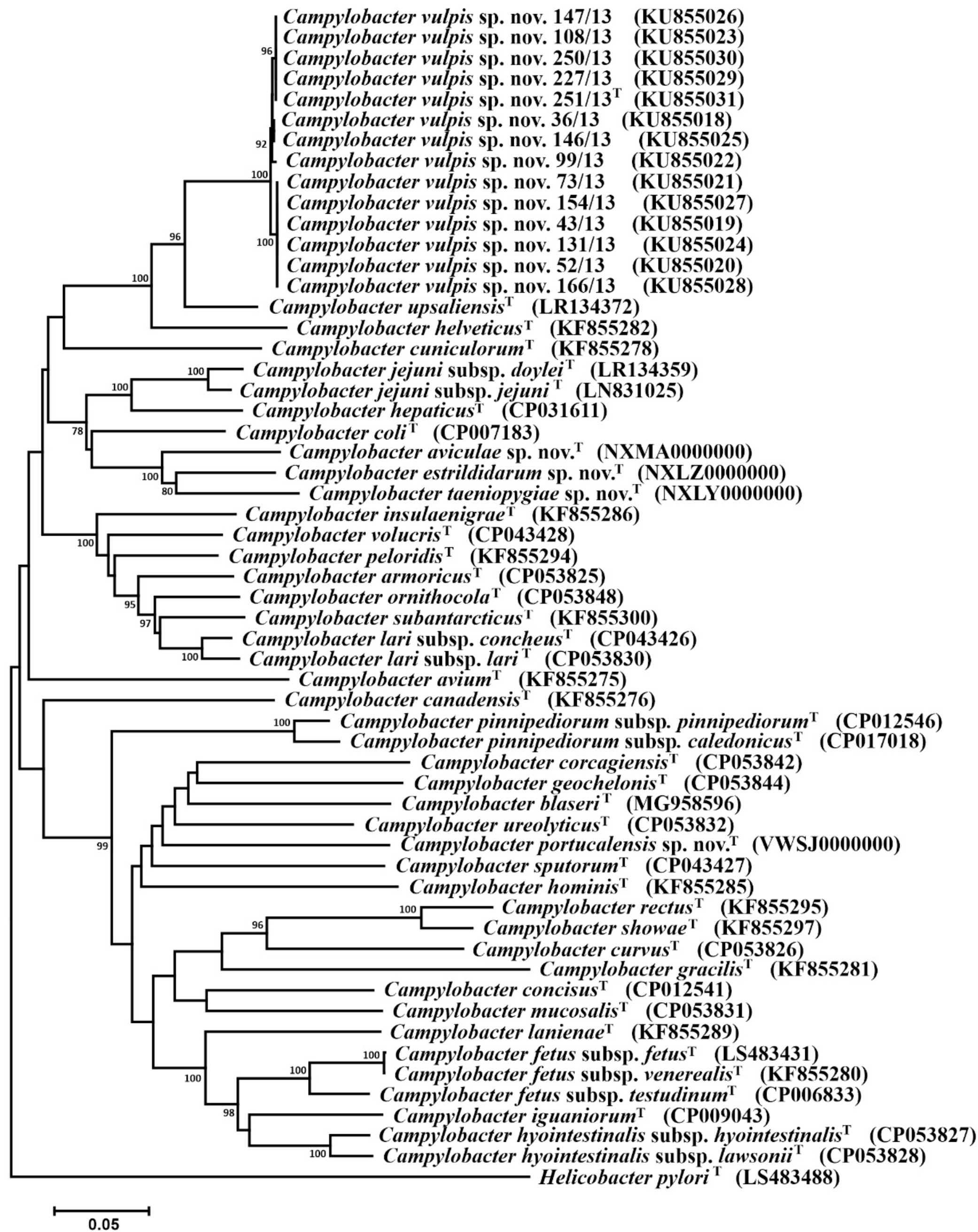


Figure 4: *atpA* phylogenetic tree representing *Campylobacter vulpis* sp. nov. and the *Campylobacter* type strains. The evolutionary history was inferred using the Neighbor-Joining method [45]. The percentage (>75%) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [22] and are in the units of the number of base substitutions per site.

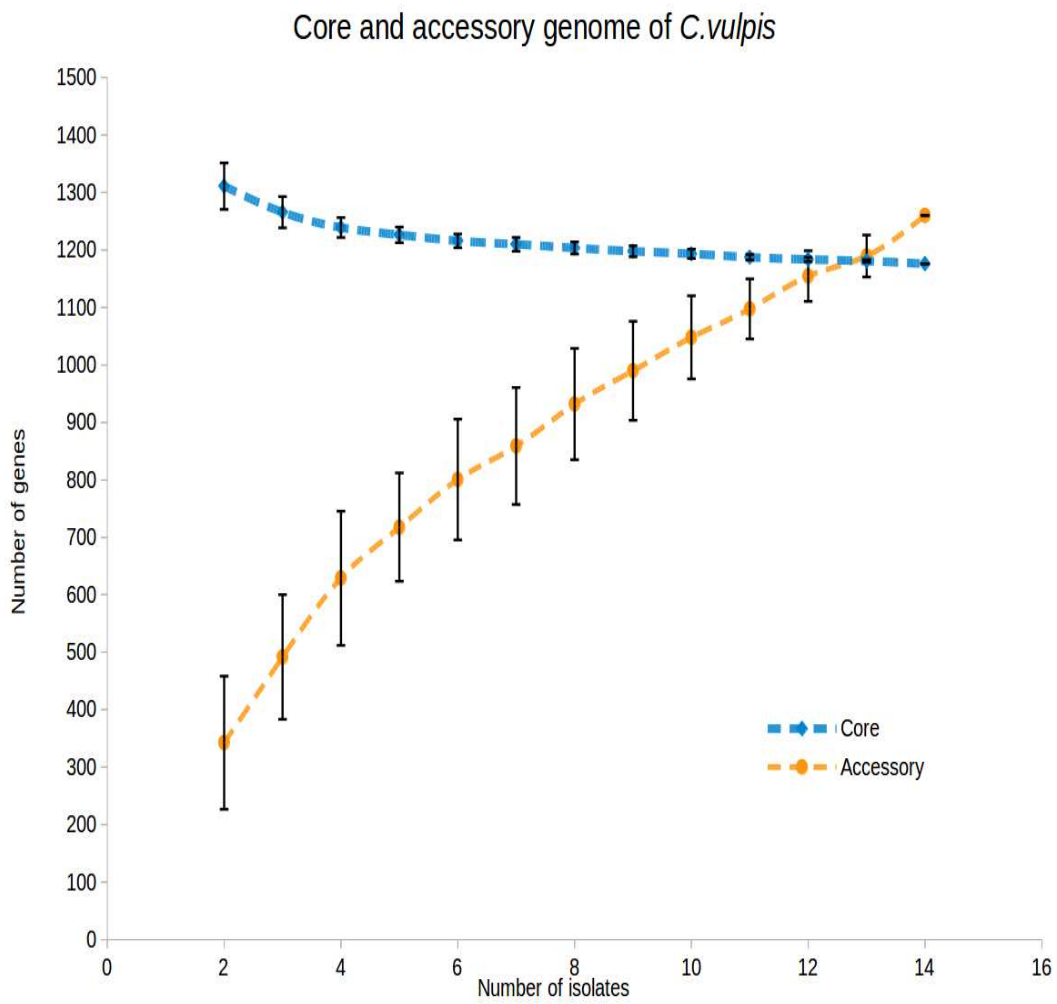


Figure 5. Core and accessory genome of *C. vulpis* sp. nov. Core genome, blue. Accessory genome, orange. The number of isolates considered is indicated on the x axis, while the number of genes is represented on the y axis. Error bars are used to indicate the standard deviation.

Table 1. Phenotypic characteristics of *Campylobacter* species

Characteristic	*vulpis* sp. nov. (n = 14)	*upsaliensis*	*helveticus*	*armoricus*	*aviculae*	*avium*	*blaseri*	*canadensis*	*coli*	*concisus*	*corcagiensis*	*cuniculorum*	*curvus*	*estrildidarum*	*fetus fetus*	*fetus testudinum*	*fetus venerealis*	*geochelonis*	*gracilis*	*hepaticus*	*hominis*	*hyointestinalis hyointestinalis*
Oxidase	+	+	+	+	+	+	+	+	+	v	+	+	+	+	+	+	+	+	-	+	+	+
Catalase	-	-	-	+	-	w	+	v	+	-	+	+	-	(-)	+	+	+	+	v	+	-	+
Urease	-	-	-	+	-	-	+	v	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Alkaline phosphatase	v	-	-	-	-	-	+	-	-	(+)	+	-	v	-	-	ND	-	-	-	ND	-	-
Hydrolysis of:																						
Hippurate	-	-	-	-	+	+	-	-	-	-	-	-	(-)	(+)	-	-	-	-	-	(+)	-	-
Indoxyl acetate	+	+	+	-	-	+	+	-	+	-	v	+	v	-	-	-	-	-	(+)	+	-	-
Reduction of:																						
Nitrate	+	+	+	-	v	+	+	v	+	(-)	(+)	+	+	v	+	+	(+)	+	(+)	v	v	+
Selenite	+	+	-	v	ND	-	ND	ND	+	(-)	ND	-	-	ND	(+)	ND	(-)	-	-	ND	-	+
TTC	-	v	-	v	ND	-	ND	ND	+	-	-	v	v	ND	-	+	-	-	-	ND	-	(-)
H_2S production (TSI)	-	-	-	ND	ND	-	+	v	-	-	+	-	(-)	ND	-	-	-	-	-	-	-	+
α-haemolysis	+	+	+	-	ND	-	-	-	(-)	(-)	-	+	(-)	ND	-	ND	v	-	-	-	-	v
Growth at/in/on:																						
25 °C (microaerobic)	-	-	-	+	-	-	+	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-
30 °C (microaerobic)	-	+	v	+	ND	-	+	-	-	(+)	+	-	+	ND	+	+	+	+	(+)	-	-	+
37 °C (microaerobic)	+	+	+	+	+	+	+	+	+	+	+	+	v	+	+	+	+	+	-	+	+	+
42 °C (microaerobic)	+	+	+	+	+	+	+	+	+	(+)	+	(+)	v	+	(+)	v	-	-	v	+	(-)	+
37 °C (anaerobic)	-	-	-	+	ND	-	+	+	-	+	+	-	+	ND	(-)	+	v	+	+	-	+	-
37 °C (aerobic)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CCDA	+	+	+	ND	ND	-	-	+	+	(-)	ND	(+)	(+)	ND	+	ND	+	+	v	ND	ND	+
Glycine (1% w/v)	+	+	v	+	(-)	-	w	v	(+)	(-)	+	-	+	(+)	+	+	(-)	+	+	+	+	+
NaCl (2% w/v)	-	-	(-)	-	ND	-	ND	-	-	(-)	+	-	v	ND	-	ND	-	+	v	-	ND	-
Bile (1% w/v)	+	+	+	ND	ND	v	ND	ND	(+)	-	+	ND	-	ND	+	ND	+	ND	-	+	ND	+
Requirement for H_2	-	-	-	-	ND	v	-	-	-	+	-	-	+	ND	-	-	-	-	+	-	+	v
Resistance to:																						
Nalidixic acid (30 µg)	-	-	-	-	-	-	-	v	-	(+)	+	v	+	(-)	+	ND	v	+	v	v	v	+
Cephalothin (30 µg)	-	(-)	-	+	+	+	-	-	+	-	ND	(+)	-	+	-	ND	-	-	-	(+)	-	(-)

Characteristic	*hyointestinalis lawsonii*	*iguaniorum*	*insulaenigrae*	*jejuni doylei*	*jejuni jejuni*	*lanienae*	*lari concheus*	*lari lari*	*mucosalis*	*ornithocola*	*peloridis*	*pinnipediorum caledonicus*	*pinnipediorum pinnipediorum*	*portucalensis*	*rectus*	*showae*	*sputorum*	*subantarcticus*	*taeniopygiae*	*ureolyticus*	*volucris*
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	+	+	+	+	+
Catalase	+	+	+	(+)	+	+	+	+	-	+	+	-	+	-	(-)	v	v*	+	+	(-)	+
Urease	-	-	-	-	-	-	-	v†	-	+	ND	+	+	-	-	-	v*	-	-	+	-
Alkaline phosphatase	(-)	ND	ND	-	-	+	ND	-	(+)	-	ND	ND	ND	ND	-	-	-	ND	-	-	-
Hydrolysis of:																					
Hippurate	-	-	-	+	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Indoxyl acetate	-	-	-	+	(+)	-	ND	(-)	-	-	ND	-	-	-	+	v	-	-	-	(-)	-
Reduction of:																					
Nitrate	+	+	+	-	+	+	+	+	(-)	v	ND	+	+	-	+	+	(+)	+	v	+	+
Selenite	+	ND	ND	-	(+)	ND	ND	v	(-)	ND	ND	ND	ND	ND	-	-	v	-	ND	-	+
TTC	-	ND	ND	v	(+)	ND	ND	(+)	-	(-)	ND	ND	ND	ND	-	-	-	ND	ND	-	-
H_2S production (TSI)	+	+	-	-	-	-	ND	-	+	-	ND	+	+	-	-	v	+	-	ND	-	-
α-haemolysis	v	+	ND	+	+	+	ND	+	-	-	ND	+	+	ND	+	+	+	+	ND	v	ND
Growth at/in/on:																					
25 °C (microaerobic)	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
30 °C (microaerobic)	+	+	-	-	(+)	-	-	+	+	-	-	+	+	ND	(-)	+	(+)	-	ND	+	-
37 °C (microaerobic)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	v	+	+	+	+	+
42 °C (microaerobic)	+	-	-	-	+	+	+	+	+	+	+	-	-	+	(-)	v	+	+	+	v	+
37 °C (anaerobic)	+	+	-	-	-	+	ND	-	+	+	ND	+	+	w	+	+	+	+	ND	+	+
37 °C (aerobic)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CCDA	+	+	ND	+	+	ND	+	+	+	ND	+	-	-	ND	-	+	(+)	ND	ND	+	ND
Glycine (1% w/v)	v	+	+	(-)	(+)	-	+	+	v	+	+	-	v	v	+	v	+	(+)	-	+	-
NaCl (2% w/v)	-	ND	-	-	-	-	+	(+)	(+)	ND	(+)	ND	ND	-	v	+	+	+	ND	+	-
Bile (1% w/v)	ND	ND	ND	+	+	ND	ND	ND	(+)	ND	ND	ND	ND	ND	-	-	v	+	ND	v	w
Requirement for H_2	v	-	ND	-	-	-	ND	-	+	-	ND	-	-	-	+	+	-	ND	ND	+	ND
Resistance to:																					
Nalidixic acid (30 µg)	+	+	+	-	-	+	-	v	(+)	ND	(+)	-	-	ND	(+)	-	(+)	+	-	-	+
Cephalothin (30 µg)	-	-	+	-	(+)	+	+	+	(-)	ND	(-)	-	-	ND	-	-	-	-	+	-	+

†: UPTC strains are urease positive; *: bv. fecalis strains are catalase positive, bv. paraureolyticus strains are urease positive; +, 90-100% positive; (+), 75-89% positive; v, 26-74% positive; (-), 11-25% positive; -, 0-10% positive; w, weak growth; ND, not determined; CCDA, charcoal cefoperazone deoxycholate agar; TTC, 2,3,5-triphenyltetrazolium chloride; TSI, triple sugar-iron agar. Data for reference taxa were taken from Piccirillo et al. [41], Van et al. [50], Cáceres et al. [9], Gilbert et al. [17], Boukerb et al. [6], Silva et al. [46], and Bryant et al. [8].

Table 2. Average amino acid and nucleotide identities among the *C. vulpis* sp. nov. strains and related *Campylobacter* taxa.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
***C. vulpis* sp. nov. 251/13^{T}**	**1**	---	99	99	99	99	99	100	99	99	100	99	100	99	100	90	85	74	75	74
***C. vulpis* sp. nov. 36/13**	**2**	99	---	99	99	99	99	99	99	100	99	99	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 43/13**	**3**	100	99	---	100	100	99	99	100	99	99	100	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 52/13**	**4**	100	99	100	---	99	99	99	100	99	99	100	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 73/13**	**5**	100	99	100	100	---	99	99	99	99	99	100	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 99/13**	**6**	100	99	100	100	100	---	99	99	99	99	99	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 108/13**	**7**	100	99	100	100	100	100	---	99	99	100	99	100	99	100	90	85	74	74	74
***C. vulpis* sp. nov. 131/13**	**8**	100	99	100	100	100	100	100	---	99	99	99	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 146/13**	**9**	99	100	99	99	99	99	100	99	---	99	99	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 147/13**	**10**	100	99	100	100	100	100	100	100	99	---	99	100	99	100	90	85	74	74	74
***C. vulpis* sp. nov. 154/13**	**11**	100	99	100	100	100	100	100	100	99	100	---	99	99	99	90	85	74	74	74
***C. vulpis* sp. nov. 166/13**	**12**	100	99	100	100	100	100	100	100	99	100	100	---	99	100	90	85	74	74	74
***C. vulpis* sp. nov. 227/13**	**13**	100	99	100	100	100	100	100	100	99	100	100	100	---	99	90	85	74	74	74
***C. vulpis* sp. nov. 250/13**	**14**	100	99	100	100	100	100	100	100	99	100	100	100	100	---	90	85	74	74	74
***C. upsaliensis* NCTC 11541^{T}**	**15**	95	95	95	95	95	95	95	95	95	95	95	95	95	95	---	85	74	74	74
***C. helveticus* ATCC 51209^{T}**	**16**	91	91	91	91	91	91	91	91	91	91	91	91	91	91	91	---	75	74	74
***C. jejuni jejuni* NCTC 11351^{T}**	**17**	81	81	81	81	81	81	81	81	81	81	81	81	81	81	81	82	---	96	84
***C. jejuni doylei* NCTC 11951^{T}**	**18**	81	80	81	81	81	81	81	81	80	81	81	81	80	81	81	81	98	---	84
***C. coli* ATCC 33559^{T}**	**19**	81	81	81	81	81	81	81	81	81	81	81	81	81	81	81	82	90	90	---

Average amino acid identity values are on the bottom left, and average nucleotide identity values are on the upper right. The average nucleotide identity (ANIb) values were calculated using JSpecies (v. 1.2.1)

Table 3. Description of *Campylobacter vulpis* sp. nov.

Genus name	*Campylobacter*
Species name	*Campylobacter vulpis*
Specific epithet	*vulpis*
Species status	sp. nov.
Species etymology	(*vulpis.* L. gen. n. *vulpis* of a fox)
Description of the new taxon and diagnostic traits	Cells are Gram-negative, sigmoid to allantoid in shape, 0.3-0.4 µm in width and 1.2-3.0 µm in length with a single flagellum at both poles and are motile with characteristic darting movements when observed by dark field microscopy. Cells appear coccoid after 5-6 days of incubation or when exposed to air. After incubation on Nutrient agar [amended with 5% (v/v) sheep blood] in a microaerobic atmosphere at 37 ± 1°C for 48 h, colonies appear 2-3 mm in diameter, α-haemolytic, grey, translucent, flat with an irregular edge, and show a tendency to spread along the direction of the streak and to swarm and coalesce. Strictly microaerophilic. Able to grow at 37 and 42 °C, but not at 25 and 30 °C or under anaerobic and aerobic conditions. Hydrogen is not required for growth. All isolates are oxidase positive, and catalase and urease negative. Hippurate is not hydrolysed, while all strains hydrolyse indoxyl acetate. Half of the isolates are alkaline phosphatase positive. Hydrogen sulfide is not produced on TSI agar. All strains are unable to reduce triphenyl tetrazolium chloride (TTC), while most reduce nitrate and selenite. All strains grow on Nutrient agar without blood and on CCDA, but not on MacConkey agar. Growth occurs in the presence of 1% (w/v) bile, but not in the presence of 2% (w/v) NaCl. Most strains grow in presence of 1% (w/v) glycine. Strains are susceptible to nalidixic acid (30 µg) and to cephalothin (30 µg) by disc diffusion tests. Pathogenicity is unknown. The type strain 251/13^T (=CCUG 70587^T, =LMG 30110^T) was isolated from the caecal contents of a wild red fox (*Vulpes vulpes*) in northern Italy in 2013. The DNA G+C content of the type strain is 34.62 mol %.
Country of origin	Italy
Region of origin	Emilia Romagna
Source of isolation	*Vulpes vulpes* – cecal content
Sampling date (dd/mm/yyyy)	17/06/2013
Latitude (xx°xx′xx″N/S)	44°33'10.5" N
Longitude (xx°xx′xx″E/W)	11°26'41.762" E
Altitude (meters above sea level)	28 m
16S rRNA gene accession nr.	KU855045
Genome accession number [RefSeq; EMBL; …]	CP041617
Genome status	Complete
Genome size	1,645 kbp
GC mol%	34.62 GC mol%
Number of strains in study	14
Source of isolation of non-type strains	*Vulpes vulpes* – cecal content
Designation of the Type Strain	251/13^T
Strain Collection Numbers	CCUG 70587^T; LMG 30110^T