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This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version: Parisi, A., Chiara, M., Caffara, M., Mion, D., Miller, W.G., Caruso, M., et al. (2021). Campylobacter vulpis sp. nov. isolated from wild red foxes. SYSTEMATIC AND APPLIED MICROBIOLOGY, 44(3), 1-11 [10.1016/j.syapm.2021.126204].

Availability: This version is available at: https://hdl.handle.net/11585/832805 since: 2023-11-21

Published:

DOI: http://doi.org/10.1016/j.syapm.2021.126204

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CAMPYLOBACTER VULPIS SP. NOV. ISOLATED FROM WILD RED FOXES

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1 ABSTRACT

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

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

22 INTRODUCTION

23 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 24 domesticated and wild birds [21]. *Campylobacter* strains are routinely isolated from the fecal 25 samples and rectal swabs of domestic dogs, both healthy and diarrheic [1, 19]. One of the 26 primary Campylobacter colonizers of domestic dogs is Campylobacter upsaliensis [1, 21]. 27 The related species, Campylobacter helveticus [47], is also recovered from domestic dogs, 28 although at a lower frequency [4, 14]. In addition to C. upsaliensis and C. helveticus, C. 29 jejuni, C. coli, C. lari and C. hyointestinalis also colonize domestic dogs [19], along with at 30 least nine other Campylobacter species (i.e., C. showae, C. concisus, C. fetus, C. gracilis, C. 31 mucosalis, C. rectus, C. sputorum, C. ureolyticus and C. volucris) at a lower reported 32 frequency [11, 19], suggesting that domestic dogs are host to a wide variety of 33 34 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 35 human health concern due to the close contact of dogs and humans. Most of the reported 36 canine-associated epidemiology of Campylobacter pertains to pet and sheltered animals. 37 Therefore, data on the prevalence of *Campylobacter* within wild canid populations is limited, 38 although C. upsaliensis has been recovered from coyotes [33]. 39

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*

From January to July 2013 the caecal contents collected from a total of 45 wild red foxes (26 50 females and 19 males, 12 subadults <1 year and 33 adults) were examined for *Campylobacter* 51 spp. and Helicobacter spp. Foxes were shot during population control programs in the 52 53 province of Bologna. For this survey, only animals dead ≤ 12 hours and kept at refrigeration temperature until necropsy were selected. Bacterial strain isolations were performed on 54 55 Nutrient sheep-blood agar [Nutrient Broth No 2 (Oxoid) amended with 1.5% (w/v) Bacto Agar (Difco) and 5% (v/v) sheep blood], using the filter technique of Steele and McDermott 56 that was modified as previously described [52], and on selective media [Campylobacter 57 58 Selective Agar (Skirrow) and Campylobacter CAT Agar (Oxoid)]. Plates were incubated at $37 \pm 1^{\circ}$ C for 72 h in a microaerobic atmosphere, with hydrogen, obtained by the gas 59 replacement method, using an anaerobic gas mixture (10% H₂, 10% CO₂, 80% N₂) as 60 described [5]. Pure cultures were obtained after dilution and repeated sub-culturing onto 61 plates of Nutrient sheep-blood agar never older than 7 days. 62

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64 Preliminary taxonomic identification and MLST typing

Genomic DNA was purified using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) and amplified using a *Campylobacter* genus-specific PCR [28] and a species-specific PCR for *C. upsaliensis* and *C. helveticus* as previously described [27]. Based on a preliminary identification of *C. upsaliensis*, the isolates were typed by multi-locus sequence typing (MLST) according to published protocols [31], with the PCR amplifications performed as described elsewhere (https://pubmlst.org/organisms/campylobacter-nonjejunicoli/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 alleles and sequence types (STs) were assigned following submission of the DNA sequences 73 jejuni/coli Campylobacter PubMLST 74 and profiles to the non database (http://pubmlst.org/campylobacter/). 75

Concatenated sequences representing all C. upsaliensis sequence types within the 76 Campylobacter PubMLST database were downloaded from PubMLST and combined with 77 sequences representing the profiles from this study. A concatenated sequence based on the C. 78 upsaliensis MLST gene get was also extracted from the type strain genome of C. helveticus. 79 80 These sequences were imported into BioNumerics and aligned using the Fast algorithm. Within BioNumerics, a Neighbor-joining dendrogram was constructed from the aligned 81 profile sequences. A minimum spanning tree (MST) was constructed based on the sequence 82 83 distances between the concatenated profile sequences and using the default priority rules, 'Permutation resampling' resampling strategy and 'Highscore summary' methods. MST 84 nodes were color-coded within BioNumerics according to taxon. 85

86

87 Biochemical and microscopic characterization

The 14 isolates were tested for the following phenotypic traits: oxidase, catalase, urease and 88 alkaline phosphatase activity; hydrolysis of hippurate and indoxyl acetate; reduction of 89 nitrate, selenite and 2,3,5-triphenyltetrazolium chloride (TTC); H₂S production on triple 90 sugar iron agar; α-haemolysis; growth at 25 °C, 30 °C, 37 °C and 42 °C; growth under 91 aerobic, microaerobic and anaerobic conditions; growth on CCDA and on media amended 92 with 1% (w/v) glycine, 2% (w/v) NaCl or 1% (w/v) bile. Resistance to nalidixic acid and 93 94 cephalothin was also determined. All tests were performed using standard methods as previously described [35-39] and were conducted at least twice. 95

Morphological characteristics of three of the novel strains were determined using transmission electron microscopy. Cells grown on Nutrient Broth No.2 (Oxoid) with 1.5% of Bacto Agar (Difco) and 10% of sheep blood for 48 h were gently suspended in 0.1 M phosphate-buffered saline (PBS) at a concentration of about 108 cells per ml. Samples were negatively stained with 2% (w/v) phosphotungstic acid (pH 6.5) for 30 s. The specimens were 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 laser was used to analyze spectra over a mass range of 2,000-20,000 Da. All the isolates and 105 C. upsaliensis ATCC 43954^{T} were processed according to the manufacturer's instructions. A 106 sterile wooden tip was used to pick an isolated bacterial colony freshly grown on Mueller-107 Hinton agar (Oxoid) supplemented with 5% sheep blood for 48 h at 37 °C under 108 microaerobic conditions. A thin film in five replicates was then smeared onto a MALDI 96-109 target polished steel plate (Direct Transfer procedure). Microbial films were overlaid directly 110 with 1.0 μ l of a 10 mg/ml α -cyano-4-hydroxycinnamic acid (MALDI-TOF HCCA) matrix 111 solution. The sample-matrix mixture was dried at room temperature and subsequently 112 inserted into the system for data acquisition. The spectra were constructed based on processed 113 114 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 presented as one main spectrum. A sum spectrum was acquired by summing the laser shots. 116 Quality controls were internally calibrated using the *Escherichia coli* DH5a supplied by 117 Bruker Daltonics, following the same procedure. The data were processed automatically by 118 the instrument software, and the spectra were compared with reference libraries for bacterial 119 identification matching. Spectra were analyzed using MBT Compass 4.1.70.1 database 120

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

128

129 *rDNA*, atpA and core gene phylogenetic analysis

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

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

then concatenated alphabetically and a Neighbor-Joining dendrogram was constructed inGeneious Prime using the Tamura-Nei genetic distance model.

148

149 Whole genome sequencing and annotation

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

171 Annotation of the type strain genome was performed manually. Putative coding sequences, ribosomal loci and tRNA/transfer-messenger RNA (tmRNA) genes were identified using 172 GeneMark, RNAmmer and ARAGORN [3, 24, 26] respectively. The start point of each 173 coding sequence was curated manually within Artemis (v.16) [44]. Gene function was 174 initially assigned following a BLASTP comparison of the 251/13^T proteome against the 175 proteomes derived from the other annotated *Campylobacter* type strains. Annotation was 176 further refined by identification of Pfam motifs [13] and a BLASTP analysis that utilized the 177 larger NCBI non-redundant (nr) protein database. Annotation of the thirteen draft genomes 178 179 was performed by means of the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [49] upon submission of the genomes to GenBank. 180

181

182 Whole-genome analyses

Analyses based on average nucleotide and amino acid identities and in silico DNA-DNA 183 hybridization were used to determine if the new isolates belonged to any of the previously-184 characterized species of Campylobacter. The average nucleotide identity based on BLAST 185 (ANIb) analysis was performed using JSpecies (v. 1.2.1) [42]. The data set analyzed included 186 the complete $251/13^{T}$ genome and the *Campylobacter* type strain genomes. The proteomes of 187 the same taxa used in the ANI analysis were compiled into a single FASTA-formatted 72,437 188 protein amino acid sequence file. An all-against-all BLASTP [2] analysis was performed 189 190 using this composite proteome file. The BLASTP output was subsequently analyzed with BlastPTrimmer14 [34] using 35% sequence similarity and 75% alignment length as input 191 parameters, which identified the proteins conserved among the 43 taxa and generated pair-192 wise average amino-acid identity (AAI) values using the core protein set. In silico DNA-193 DNA hybridization (isDDH) values were determined using the Genome-to-Genome Distance 194 Calculator 2.1 (GGDC; https://ggdc.dsmz.de/ggdc.php#; accessed Sept 2020) [30]. ANI, AAI 195

and isDDH analyses were also performed on a 28-strain set that included an additional 25 C. 196 vulpis, C. upsaliensis and C. helveticus strains. In addition to the three complete C. vulpis, C. 197 upsaliensis and C. helveticus type strain genomes and the thirteen C. vulpis draft genomes 198 199 sequenced here, ten C. upsaliensis genomes and two C. helveticus genomes were downloaded from NCBI. ANIb, AAI and isDDH analyses were performed as described above, using a 200 FASTA-formatted 48,744 protein amino acid sequence file for the AAI analysis. Whole-201 genome based average nucleotide identity (gANI) analysis was performed using the JGI 202 Microbial Species Identifier (MiSI; https://ani.jgi.doe.gov/html/home.php?; accessed Mar 203 204 2021 [51]) and FASTA-formatted coding sequence files. The second isDDH analysis used a mixture of draft and complete genomes; therefore, Formula 2 (recommended for draft 205 genomes as it is independent of genome length) was used throughout both isDDH analyses to 206 207 maintain consistency.

208

209 Clusters of Orthologous Genes (COGS) and Rarefaction Analyses

Clusters of reciprocal best BLASTP matches [putative Clusters of Orthologous Genes 210 (COGs)] were established using all-against-all BLASTP searches, which employed the 211 BLOSUM80 matrix and accepted only the best reciprocal matches with E-values $\leq 1 \times 10^{-5}$, 212 where "second-best" matches produced bit scores < 90% of those associated with the best 213 matches, in order to avoid confounding effects deriving from recent duplications and 214 215 paralogous genes. The estimated size of the core and accessory genome, based on the COGs identified by the method described above, were recorded for all possible combinations of the 216 2 to 14 genomes included in this study. The core genome of the genus *Campylobacter* was 217 determined by applying the same method to a collection of 38 Campylobacter type strain 218 proteomes, with the inclusion of $251/13^{T}$ as the type strain of the putative new species. Plots 219 were prepared showing mean and standard deviation of these combinations. The scripts used 220

for the identification of COGs and execution of rarefaction analysis for the estimation of the size of the core and accessory genomes of the studied isolates are available at https://github.com/cvulpispaper/compute_aai_and_cogs.

224

225 Identification of "plasmid-like" sequences

The complete collection of plasmid sequences included in the PLSDB database [16] was retrieved from https://ccb-microbe.cs.uni-saarland.de/plsdb/plasmids/download/?zip (accessed Sept 2020). Sequence similarity searches against the PLSDB were performed using BLASTN, as available from the BLAST+ suite [10].

230

231 Species-specific PCR

In order to define a diagnostic method for the rapid detection and identification of the 232 putative new species, specific PCR primers targeting the *atpA* gene were designed: 233 atp_FW18 5'-TGC CGC TTT ACA ATA TCT CGC T-3' and atp_Rev405 5'-CCC CAC 234 ACG CGA AAC AGA CAA G-3'. Amplification parameters were as follows: 30 cycles of 235 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 5 236 min and followed by an extended elongation step at 72 °C for 7 min. To assess the sensitivity 237 and specificity of this PCR method, all 14 strains and a selection of strains representing 238 different species of *Campylobacter* were analyzed using the specified conditions. 239

240

241 Data availability

The GenBank accession numbers for the 16S rRNA gene and *atpA* sequences of strains 36/13, 43/13, 52/13, 73/13, 99/13, 108/13, 131/13, 146/13, 147/13, 154/13, 166/13, 227/13, 250/13 and 251/13^T are KU855032-KU855045 (16S) and KU855018-KU855031 (*atpA*). The complete sequence of the type strain $251/13^{T}$ has been deposited in GenBank [CP041617

(chromosome) and CP041618 (megaplasmid pVULP)]. The draft genome sequences of the 246 other strains have been deposited in GenBank with the following accession numbers: 247 (36/13), VJYW0000000 (43/13), VJYU00000000 (52/13), VJYO0000000 248 VJYY00000000 (73/13),VJYM0000000 (99/13), VJYT00000000 (108/13),249 VJYV00000000 (131/13),VJYN0000000 (146/13),VJYS0000000 (147/13),250

VJYQ00000000

(166/13),

VJYP0000000

(227/13),

252 VJYR0000000 (250/13).

VJYX00000000

253

251

254 **RESULTS AND DISCUSSION**

255 Morphology and phenotypic characterization

(154/13),

After 48 h of incubation at 37 \pm 1°C, 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. 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 one bipolar unsheathed flagellum (Fig. 1), and 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.

All 14 isolates from red foxes were identified initially as C. upsaliensis according to the 262 results of a *Campylobacter* genus-specific PCR and a species-specific PCR for *C. upsaliensis* 263 and C. helveticus. Thus, it was not unexpected that results of the standard biochemical and 264 growth tests showed a strong similarity between the composite phenotypic profile observed 265 for the 14 fox isolates and the phenotypic profile reported previously for C. upsaliensis 266 (Table 1). Nevertheless, the 14 fox isolates could be unambiguously distinguished from C. 267 upsaliensis by their inability to grow at 30 °C under microaerobic conditions (Table 1). 268 Additionally, alkaline phosphatase activity is variable within our strain set but negative in C. 269 upsaliensis, and TTC reduction is negative in our strain set but variable in C. upsaliensis. 270

Although the profile from our strain set is also highly similar to the profile reported for *C*. *helveticus*, they are clearly distinguished from *C*. *helveticus* by their ability to reduce selenite (Table 1).

274

275 MALDI-TOF analysis

We determined if our 14 fox isolates could also be distinguished from *C. upsaliensis* using MALDI-TOF MS. MALDI-TOF MS has been used in previous descriptions of novel *Campylobacter* taxa; *Campylobacter fetus* subsp. *testudinum* could not be distinguished from *C. fetus* subsp. *fetus* or *C. fetus* subsp. *venerealis* by standard phenotypic tests but were clearly distinct from those subspecies based on MALDI-TOF MS profiles [15], and MALDI-TOF MS analysis was used as additional supporting evidence in the description of *Campylobacter armoricus* [6].

MALDI-TOF MS spectra were generated for the 14 strains. The result "No Organism 283 Identification Possible" was obtained by comparing these experimental mass spectra against 284 the data contained in the Compass database used in this study. The average mass spectra of 285 all acquired experimental data for *C. upsaliensis* NCTC 11541^T and the 14 isolates overlap 286 across the entire mass range, although some differences are noticeable in the protein profiles. 287 In the spectral regions between m/z 8200-8600 and m/z 10180-10260, there are characteristic 288 peaks for both species (Supplementary Fig. 1). Specific peaks at m/z values of 8376, 8424 289 and 10201 for C. upsaliensis NCTC 11541^T and m/z values of 8510 and 10227 for two fox 290 strains (251/13^T; 73/13), as monoprotonated molecules, were identified (Supplementary Fig. 291 1). The same proteins are revealed in the mass spectra as doubly-charged ions, as 292 demonstrated by the replication of the same profiles in the spectral range between m/z 4180-293 4270 and 5095-5120 (Supplementary Fig. 1). The results of the Variation Coefficient tests, in 294 terms of standard deviation of intensity values, and the ROC curves, in terms of Area Under 295

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

303

304 C. vulpis *multi-locus sequence typing*

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

313

314 16S rDNA, atpA and core gene phylogenetic analysis

Alignment of the 16S rDNA sequences from *C. vulpis* and the *C. upsaliensis* type strain indicated identity (0% sequence distance) between the *C. vulpis* strains, with near identity (99.8% similarity) to the 16S rDNA sequence of the *C. upsaliensis* type strain. This is reflected in the dendrogram constructed from the aligned sequences (Fig. 3). However, similarity values between the *C. vulpis* 16S rDNA loci and those of five additional *C.* *upsaliensis* strains range from 98.5% to 99.8% (data not shown). High similarity between the 16S rDNA loci of related taxa has been reported previously in *Campylobacter*; the 16S rDNA sequences of three novel species recovered from Zebra finches are 99% similar [8]. Nevertheless, the neighbor-joining tree demonstrates that our strains and the *C. upsaliensis* type strain form a well-supported (92% bootstrap) clade that is separate from the rest of the *Campylobacter* species included in the dataset (Fig. 3).

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

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

334

335 Whole genome sequencing and analysis

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

sequencing (Supplementary Table 1). The approximate sizes of these genomes range from 345 1,574 to 1,724 kbp with G+C contents ranging from 34.4 to 34.7%. These %G+C values are 346 similar to those reported for C. upsaliensis (i.e., 34-35%) and are within the range reported 347 for other members of the genus Campylobacter (29–47 %) [12]. BLASTN analysis of the C. 348 vulpis draft genomes, using pVULP as a query sequence, indicated the potential presence of 349 plasmids in nine of the thirteen strains (Supplementary Table 1); these results were confirmed 350 by BLAST sequence similarity searches against the PLSDB database, a specialized database 351 containing the complete sequences of more than 18,000 plasmids. However, it is possible that 352 353 these plasmid sequences in the draft genomes are located in genomic islands rather than on extrachromosomal elements; in addition to pVULP, P-type type IV conjugative transfer 354 system encoding genes are also located within the 19 and 33 kbp genomic islands on the 355 251/13^T chromosome. Therefore, determination of the plasmid content of the draft genomes 356 may require genome closure. 357

Rarefaction analysis based on the *C. vulpis* genomes sequenced in this study (Fig. 5), indicates that the core genome of *C. vulpis* is 1176 genes and accounts for more than 70% of the average *C. vulpis* gene content. Furthermore, the core genome curve illustrated in Figure 5 suggests that the size of the core genome is not likely to be affected substantially by the sequencing of additional genomes. However, the accessory genome presently contains 1260 genes. This is clearly a minimum value (Fig. 5), suggesting that currently available data offer only a partial representation of the pan-genome of *C. vulpis*.

365

366 Whole genome comparisons

Average nucleotide identity based on BLAST (ANIb) analysis indicated that the *C. vulpis* type strain showed the highest levels of sequence identity with the *C. upsaliensis* type strain, 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 of 90.1% (Table 2, Supplementary Table 2B). The average ANIb value between C. vulpis 371 strains was 99.2% (Supplementary Table 2B). Importantly, ANIb values determined for every 372 isolate considered in this study are well below the 94-95% cut-off that has been recently 373 recommended for the discrimination of bacterial species [18, 23, 42]. Moreover, the average 374 pairwise C. vulpis/C. upsaliensis^T ANIb value is below the ANIb values observed for all 375 currently-recognized Campylobacter subspecies pairs: C. jejuni subspp. jejuni and doylei 376 (96.1%); C. lari subspp. lari and concheus (93.2%); C. fetus subspp. fetus/venerealis and 377 378 testudinum (91.8%); C. hyointestinalis subspp. hyointestinalis and lawsonii (94.2%); and C. pinnipediorum subspp. caledonicus and pinnipediorum (94.3%) (Supplementary Table 2A). 379 This average pairwise ANIb value is also below that observed between C. lari subsp. 380 381 concheus and C. ornithocola (91.0%; Supplementary Table 2A).

To further characterize the average nucleotide identities within C. vulpis, C. upsaliensis and 382 C. helveticus, we used whole-genome based average nucleotide identity (gANI) analysis in 383 addition to ANIb on an expanded strain set that included all 14 C. vulpis strains, 11 C. 384 upsaliensis strains and 3 C. helveticus strains. Each method calculates the ANI in a different 385 fashion: ANIb cuts the query genome into 1020 nt fragments which are then used to search 386 against the reference genome using BLASTN [18], whereas gANI utilizes FASTA-formatted 387 coding sequence files for each genome, which are then compared using BLASTN [23]. 388 389 Although, in general, the gANI value was slightly higher than the ANIb value for each strain pair, the two ANI methods yielded very similar results (Supplementary Table 2B). 390 Interestingly, ANI analysis within C. upsaliensis identified two strains, RM3195 and NCTC 391 12264, that are more divergent than the other nine strains, with intergroup ANI values of 392 ~96.6% (Supplementary Table 2B). Nevertheless, despite this variation within C. upsaliensis, 393 both ANI methods yielded C. vulpis vs. C. upsaliensis values [90.1 \pm 0.2 (ANIb); 90.4 \pm 0.2 394

(gANI)] well below the 94-95% ANI species boundary (Supplementary Table 2B). Palmer et 395 al. [40] concluded that the various described ANI methods do not completely correlate within 396 the region of 90-100% similarity, a region that includes the proposed ANI species boundary. 397 This lack of correlation would be more critical if the calculated ANI values were in close 398 proximity to the species boundary, i.e. ~94-95%. However, the C. vulpis vs. C. upsaliensis 399 ANI values determined here, which are ~4% below the species boundary using either ANI 400 method, strongly suggest that the C. vulpis and C. upsaliensis strains comprise two distinct 401 *Campylobacter* species, a conclusion that is likely ANI method independent. 402

403 Average amino acid identity (AAI) analysis, based on the determination of pairwise similarities among the Campylobacter core protein set, indicated that the C. vulpis core 404 proteins are most similar to those of the C. upsaliensis type strain, with an average AAI of 405 95% (Table 2). Campylobacter vulpis core proteins are also similar to those of C. helveticus 406 (AAI = 91%; Table 2). AAI values between C. vulpis and the remaining Campylobacter taxa 407 were $\leq 81\%$ (Table 2, Supplementary Table 2A). AAI analysis using the expanded strain set 408 described above yielded similar results. The average pairwise AAI value between the 14 C. 409 vulpis strains and the 11 C. upsaliensis strains was 94.6% (Supplementary Table 2C), which 410 is below the intraspecific C. vulpis and C. upsaliensis values of 99.6% and 98.8%, 411 respectively (Supplementary Table 2C). Here also, AAI values between C. vulpis and C. 412 upsaliensis are below those observed within established Campylobacter subspecies pairs 413 414 (Supplementary Table 2A). Additionally, the AAI values are similar to those observed between some Campylobacter species [e.g., C. rectus and C. showae (95%), and C. 415 subantarcticus and C. lari (95%); Supplementary Table 2A]. 416

417 The estimated in silico DNA-DNA hybridization (isDDH) value between the C. upsaliensis type strain genome and the C. vulpis type strain genome is 40.1% (CI [37.7 - 42.7%]) 418 (Supplementary Table 3). Analysis of the expanded strain set yielded a similar average value

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(40.2%; Supplementary Table 2C). isDDH analysis using the 28-strain set is complicated by 420 the mixture of draft and complete genomes. Formulas 2 and 3 on the GGDC service divide 421 the high-scoring segment pairs (HSPs) by HSP length or genome length, respectively. Thus, 422 Formula 2 is recommended by the GGDC service for draft genomes of uncertain genome 423 length, and we utilized Formula 2 throughout this study to maintain consistency. Notably, the 424 variation within C. upsaliensis described above is also observed here, with isDDH values of 425 ~70% when strains RM3195 and NCTC 12264 were compared to the other nine C. 426 upsaliensis strains (Supplementary Table 2C). However, as with the ANI results, this 427 variation within C. upsaliensis did not substantially affect the pairwise C. vulpis/C. 428 upsaliensis isDDH values, although these data may indicate that further research on C. 429 upsaliensis taxonomy may be warranted. Although these isDDH data support the description 430 431 of C. vulpis as a novel Campylobacter species, the presence of incomplete genomes within the data set suggest that isDDH analysis should not be used as the sole taxonomic 432 determinant. 433

434

435 C. vulpis-*specific PCR*

To specifically amplify *C. vulpis* strains, a novel primer pair was designed that targets that *atpA* gene. Amplification of all 14 *C. vulpis* isolates produced a 409 bp PCR product, whereas the other *Campylobacter* species were PCR-negative. Additionally, the 39 *C. upsaliensis* strains characterized by Rossi et al. [43] from domestic dogs and cats were also PCR-negative. DNA sequencing of the PCR products from *C. vulpis* confirmed the specificity of the amplicons. This suggests that the *C. vulpis*-specific PCR is robust and can be used to rapidly discriminate between *C. vulpis* and *C. upsaliensis* strains.

443

444 CONCLUSIONS

Fourteen Campylobacter isolates were recovered from the cecal contents of wild red foxes in 445 Northern Italy in 2013. These strains were identified initially as C. upsaliensis by 446 Campylobacter species-specific PCR; however, MLST placed these isolates in a clade 447 separate from C. upsaliensis. MLST also identified nine sequence types within the 14 448 isolates, indicating genomic variation within the strain set. Although phenotypically similar 449 to both C. upsaliensis and C. helveticus, these 14 fox isolates could be distinguished from C. 450 upsaliensis and C. helveticus by microaerobic growth at 30 °C and reduction of selenite, 451 respectively. Discrimination of the fox isolates and C. upsaliensis could also be observed 452 453 using MALDI-TOF MS analysis. These results suggested that the 14 fox isolates, although related to C. upsaliensis and C. helveticus, were representatives of a novel species distinct 454 from other validly-described species of the genus Campylobacter. The name Campylobacter 455 *vulpis* sp. nov. was proposed, with isolate $251/13^{T}$ (=CCUG 70587^T; =LMG 30110^T) as the 456 type strain. 16S rDNA, *atp*A, core gene phylogenetic analyses and whole-genome sequence 457 comparisons, including ANI, AAI and isDDH analyses, were also performed to further 458 address the taxonomic placement of C. vulpis. atpA and core gene dendrograms placed C. 459 vulpis strains into clades related to but well separated from C. upsaliensis. Additionally, 460 pairwise C. vulpis/C. upsaliensis ANI and AAI values were below those observed for existing 461 Campylobacter subspecies pairs but consistent with other Campylobacter species pairs. 462 Taken together, these data support the designation of C. vulpis sp. nov. as a novel 463 464 Campylobacter species. A robust C. vulpis-specific PCR was developed which will permit the rapid identification of C. vulpis strains recovered from animal, environmental, or possibly 465 human clinical samples. Description of *Campylobacter vulpis* sp. nov. is presented in Table 466 3. 467

468

469 ACKNOWLEDGEMENTS

We are in debt to A. Buchicchio and A. Bianco for their technical assistance. We also thank
Emma Yee for technical assistance in sequencing the *C. vulpis* genomes. This publication
made use of the *Campylobacter* Multi Locus Sequence Typing website (https://pubmlst.org/
campylobacter/) sited at the University of Oxford [20]. The development of this site has been
funded by the Wellcome Trust.

475

476 FUNDING INFORMATION

This work was funded by University of Bologna (Italy) Grant RFO14 and by the United
States Department of Agriculture, Agricultural Research Service, CRIS project 2030-42000230-051.

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

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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.

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Figure 2. MLST-based minimum spanning tree illustrating the genotypic relationships of the 645 C. vulpis sp. nov. strains from this study and strains representing all C. upsaliensis sequence 646 types present within the PubMLST database (https://pubmlst.org/campylobacter/; accessed 647 Sept 2020). The C. helveticus MLST locus set is different from that of C. upsaliensis; thus, to 648 ensure consistency, allele sequences from the locus set used in the C. upsaliensis MLST 649 650 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 651 strains. 652

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Figure 3. 16S phylogenetic tree representing Campylobacter vulpis sp. nov. and the 654 Campylobacter type strains. The evolutionary history was inferred using the Neighbor-655 Joining method [45]. The percentage (>75%) of replicate trees in which the associated taxa 656 clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The 657 tree is drawn to scale, with branch lengths in the same units as those of the evolutionary 658 distances used to infer the phylogenetic tree. The evolutionary distances were computed 659 using the Kimura 2-parameter method [22] and are in the units of the number of base 660 661 substitutions per site.

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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.

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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.

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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 (<u>https://pubmlst.org/campylobacter/</u>; 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 ₂ S 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	- -	т 	т -	(- -	- -	- -	- -		- -	- -	-	т -	т _	(-)	v	v*	- -	- -	(-)	- -
Urease	-	т.	-	(+)	-	- -		v†	_	- -	ND	-	т -		(-)	v _	v v*	-	-	(-)	- -
Alkalina phosphatasa		- ND		-	-	-		v	- (1)	т	ND				-	-	v		-	т	-
Hydrolysis of:	(-)	ND	ND	-	-	Ŧ	ND	-	(+)	-	ND	ND	ND	ND	-	-	-	ND	-	-	-
Hippurate				Т	(+)														-		
Indoxyl acetate	-	-	-	т 	(+)	-		$\overline{()}$	-	-		-	-	-	-	-	-	-	т	$\overline{()}$	-
Reduction of:	_	-	_	Т	(+)	_	ΝD	(-)	-	_	ΝD	_	-	-	т	v	_	_	_	(-)	_
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 ₂ S production (TSI)	+	+	-	-	-	-	ND	-	+	()	ND	+	+	-	_	v	+	-	ND	_	_
a-haemolysis	v	+	ND	+	+	+	ND	+	-	_	ND	+	+	ND	+	+	+	+	ND	v	ND
Growth at/in/on:	•		112		•		1.12				1,12	•	•	112	•	•	•		112	•	T (D
$25 ^{\circ}\mathrm{C} (\mathrm{microaerobic})$	-	+	_	_	-	_	_	_	_	-	_	+	+	-	_	-	_	-	_	_	_
$30 ^{\circ}\mathrm{C}$ (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 $(1\% \text{ w/v})$	ND	ND	ND	+	+	ND	ND	ŇĎ	(+)	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
<i>C. vulpis</i> 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
<i>C. vulpis</i> 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
<i>C. jejuni jejuni</i> NCTC 11351 ^T	17	81	81	81	81	81	81	81	81	81	81	81	81	81	81	81	82		96	84
<i>C. jejuni doylei</i> NCTC 11951 ^T	18	81	80	81	81	81	81	81	81	80	81	81	81	80	81	81	81	98		84
<i>C. coli</i> 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)

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 μ m in width and 1.2-3.0 μ m 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 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 μ g) and to cephalothin (30 μ g) 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 (<i>Vulpes vulpes</i>) 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	<i>Vulpes vulpes</i> – 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

Table 3.	Description	of Campy	lobacter	vulpis sp.	nov.