

## Population genetics and characterization of *Campylobacter jejuni* isolates from western jackdaws and game birds in Finland

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1 **Population genetics and characterization of *Campylobacter jejuni* isolates in western**  
2 **jackdaws and game birds in Finland**

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12 **ABSTRACT**

13 Poultry is considered a major reservoir and source of human campylobacteriosis but the roles of  
14 environmental reservoirs, including wild birds, have not been assessed in depth. In this study, we  
15 isolated and characterized *Campylobacter jejuni* from western jackdaws (n=91, 43%), mallard  
16 ducks (n=82, 76%) and pheasants (n=9, 9%). Most of the western jackdaw and mallard duck *C.*  
17 *jejuni* isolates represented MLST sequence types (STs) that diverged from those previously  
18 isolated from human patients and various animal species, whereas all pheasant isolates  
19 represented ST-19, a common ST among human patients and other hosts worldwide. Whole-  
20 genome MLST revealed that mallard duck ST-2314 and pheasant ST-19 isolates represented  
21 bacterial clones that were genetically highly similar to human isolates detected previously.  
22 Further analyses revealed that in addition to divergent ClonalFrame genealogy certain genomic

23 characteristics, e.g. novel *cdtABC* gene cluster and T6SS of the western jackdaw *C. jejuni*  
24 isolates may affect their host-specificity and virulence. Game birds may thus pose a risk for  
25 acquiring campylobacteriosis, and therefore, hygienic measures during slaughter and meat  
26 handling warrant special attention.

## 27 **IMPORTANCE**

28 The roles of environmental reservoirs, including wild birds, in the molecular epidemiology of  
29 *Campylobacter jejuni* have not been assessed in depth. Our results showed that game birds may  
30 pose a risk for acquiring campylobacteriosis, because they had *C. jejuni* genotypes highly  
31 similar to human isolates detected previously. Therefore, hygienic measures during slaughter and  
32 meat handling warrant special attention. On the contrary a unique phylogeny was revealed for  
33 the western jackdaw isolates and certain genomic characteristics identified among these isolates  
34 are hypothesized to affect their host-specificity and virulence. Comparative genomics within  
35 STs, using wgMLST, and phylogenomics are efficient methods to analyze the genomic  
36 relationships of *C. jejuni* isolates.

## 37 **Introduction**

38 With more than 200,000 annually reported cases, *Campylobacter jejuni* continues to be the most  
39 common cause of human bacterial gastroenteritis in the European Union (EU), including Finland  
40 (1). Poultry has been recognized to be a major reservoir and source of human campylobacteriosis  
41 (1). However, in Finland, the prevalence of *Campylobacter*-positive broiler flocks has remained  
42 low since 2004, when systematic reporting started (2), while simultaneously the incidence in the  
43 human population has shown an increasing trend (incidence 69/100,000 in 2004 and 85/100,000  
44 in 2016) (3). Besides poultry, wild birds have often been shown to carry *C. jejuni*, and hence, are  
45 considered to act as wildlife reservoirs, causing fecal contamination of the environment of food  
46 production farms and possibly transmitting *Campylobacter* to domestic animals as well as to  
47 humans (4). *C. jejuni* has been found in various wild bird species (4-7), however, relatively little  
48 is known about its occurrence in western jackdaws and game birds.

49 Western jackdaw (*Corvus monedula*) belongs to the family of crows and is one of the most  
50 common bird species in some urban and agricultural surroundings in Finland, thus being also a  
51 concern from the public health perspective as a potential reservoir of zoonotic pathogens,  
52 including *Campylobacter jejuni*. The Finnish jackdaw population has increased rapidly during  
53 the last thirty years (8). Jackdaws form large flocks producing noise and considerable fecal loads  
54 that contaminate the environment, including the surroundings of food production farms and city  
55 parks. The western jackdaw is an omnivore, feeding on plants and invertebrates as well as food  
56 waste. Jackdaw flocks also feed in farmland and may cause marked agricultural damage (8).  
57 Jackdaws are partial migratory birds; adults overwinter near their nests, whereas juveniles  
58 migrate to the Southern parts of the Baltic Sea.

59 Besides natural wild game bird populations that are hunted during hunting season, several bird  
60 species, including waterfowl, pheasants, and pigeons, are bred and raised on specific farms and  
61 released for hunting purposes. Mallard duck (*Anas platyrhynchos*) is the most common game  
62 bird in Finland, with 210,000 to 300,000 birds hunted annually ([www.riista.fi/en/](http://www.riista.fi/en/)). Mallard  
63 ducks live close to the water systems and feed on plants and invertebrates. Most of the Finnish  
64 natural mallard duck population migrates in late autumn to Central Europe, but some flocks stay  
65 in Finland year-round. The hunting season lasts approximately four months (August to  
66 December), and meat is mostly consumed by individual hunters (Ministry of Agriculture and  
67 Forestry, [www.mmm.fi](http://www.mmm.fi)).

68 Pheasants (*Phasianus colchicus*) belong to the same family as partridges and are a common  
69 domesticated game bird in Europe and in the USA (9). In the UK and Ireland alone, up to 30  
70 million pheasants are hunted annually (10). These birds live typically in open spaces and fields  
71 and feed on plants, grain, seeds, insects, and invertebrates. In Finland, 15,000 to 75,000  
72 pheasants are hunted annually ([www.riista.fi/en/](http://www.riista.fi/en/)), and the meat is mostly consumed by  
73 individual hunters, but also sold to restaurants (Ministry of Agriculture and Forestry,  
74 [www.mmm.fi](http://www.mmm.fi)).

75 Multilocus sequence typing (MLST) has been an invaluable molecular typing method, providing  
76 essential knowledge about *C. jejuni* types occurring in various hosts and sources worldwide.  
77 MLST is, however, limited to the characterization and discrimination of isolates to sequence type  
78 (ST) (11-13), and thus, more accurate methods, such as whole-genome (wg) MLST, are  
79 increasingly being used (11, 14) to compare genetically related isolates in more detail and to be  
80 able to identify clones potentially originating from the same source. In previous studies, most of  
81 the STs found among wild birds, including mallard ducks (5), barnacle geese (7), starlings (15),

82 and several other bird species (16), have been considered to represent mainly host-associated  
83 STs, differing from those STs reported in human patients or domestic animals. Thus, wild birds  
84 are commonly considered to have a minor role in human campylobacteriosis. However, certain  
85 STs and generalist lineages that are common in human patients (e.g. ST-45 CC) have been  
86 detected in several wild bird species as well (5-7, 16, 17), indicating that wild birds are potential  
87 reservoirs for certain common STs also infecting humans. For example, ST-45, ST-677, and ST-  
88 267, which have been common STs in human infections also in Finland (18, 19), have been  
89 found among both blackbird and chicken isolates from Sweden (6). However, to our knowledge  
90 there have been no comprehensive wgMLST level studies, and only one study including  
91 comparative genomic analyses on wild bird isolates (American crows) has been published to date  
92 (17).

93 The aim of this study was to assess the occurrence, population genetics, and diversity of  
94 *Campylobacter* spp. in western jackdaws and game birds (i.e. mallard duck and pheasant) in  
95 Finland using whole genome sequencing. We further explored the presence of genomic features  
96 associated with virulence and antimicrobial resistance among the isolates to evaluate their  
97 importance from a public health perspective.

## 98 **Results**

### 99 **Occurrence and Multi-Locus Sequence Typing (MLST)**

100 *Campylobacter* spp. was isolated from 91 western jackdaw (43%), 79 farmed game mallard duck  
101 (79%), 9 pheasant (9%), and 3 wild mallard duck (38%) samples. All isolates were identified as  
102 *C. jejuni*.

103 Whole-genome sequencing (WGS) was successful for 87 (of 91) western jackdaw *C. jejuni*  
104 isolates. MLST showed great diversity, as 62 different sequence types (STs) were found, 46 of  
105 which were novel to the PubMLST database (pubMLST.org/campylobacter) (Supplementary  
106 Table S1). ST-1282 and ST-6460 were the most frequently found STs and also the most widely  
107 distributed both temporally and geographically. Among mallard ducks, 36 of 38 selected  
108 representative isolates (35 farmed, chosen based on PFGE screening, and 3 wild mallard duck  
109 isolates) were successfully sequenced. Altogether 16 STs were identified, 4 of which were novel  
110 to the PubMLST database. Taking into account the combined results of PFGE and MLST, the  
111 most common STs in mallard ducks were ST-2314 (n=26), ST-1299 (n=16), ST-991 (n=7), ST-  
112 2839 (n=7), and ST-995 (n=5). All of the nine pheasant isolates represented ST-19 (ST-21 CC).

### 113 **Phylogenomics**

114 A phylogenomic tree based on the core genome alignment of altogether 1261 *C. jejuni* strains  
115 (Supplementary Dataset S1.), including the ones sequenced in this study and those collected  
116 from public databases, is shown in Fig. S1. The western jackdaw isolates clustered together in a  
117 diffuse branch including also American crow and few chicken isolates and a very limited number  
118 of genomes representing sandhill crane, mallard duck and other mammals. All the other isolates  
119 even inside the branch were clearly distinct from the western jackdaw isolates which also were  
120 highly diverse. The mallard duck isolates also showed great diversity, however the majority  
121 clustered together with *C. jejuni* strains isolated from few chicken and barnacle goose. The  
122 pheasant isolates formed a more homogenous cluster and shared the same branch with a higher  
123 proportion of agricultural *C. jejuni* isolates from chicken, some bovine and other mammals.

## 124 **ClonalFrame genealogy**

125 ClonalFrame genealogy representing the phylogeny of the STs is shown in Fig. 1. ClonalFrame  
126 revealed that most of the STs occurring in western jackdaws were in their own diffuse cluster  
127 and differed from those that have been previously detected from other hosts in Finland, including  
128 other wild bird species. Exceptions were ST-4565 and ST-7841, which were clearly distinct from  
129 other western jackdaw STs and located in the same branch with the STs detected from humans,  
130 poultry, and bovines (ST-677 CC and related STs), and also ST-6460, which was a common ST  
131 among the western jackdaw isolates, but has previously been found also in poultry in Finland.

132 STs detected from mallard ducks occurred mostly in the same branches as STs detected  
133 previously from barnacle geese. Exceptions were ST-6228 and ST-7843, which were in the same  
134 cluster with western jackdaw STs, ST-995, which has previously been isolated also from poultry,  
135 and ST-6788, which was closely related to ST-1332, previously isolated from human patients  
136 and poultry. The most commonly found ST among farmed mallard ducks, ST-2314, was not  
137 closely related to any other ST previously reported in Finland.

138 ST-19 (ST-21 CC), represented by the pheasant isolates, has previously been isolated from two  
139 human patients in Finland, and it has been common in several other countries in various sources,  
140 including humans, poultry, and bovine ([www.pubMLST.org/campylobacter](http://www.pubMLST.org/campylobacter)).

## 141 **Whole-genome (wg) MLST**

142 WgMLST analysis of ST-1282, the most common ST among western jackdaw isolates, obtained  
143 from two different locations and on five separate occasions, revealed high numbers of allelic  
144 differences (62 to 439) between the isolates (Fig. 2A). The two isolates with the fewest allelic  
145 differences (n=62), CB346 and CB384, were isolated from the same town, but temporally five



146 months apart, while two isolates (e.g. CB299 and CB288) were collected from the same  
147 sampling site on successive sampling days, but they had 313 allelic differences. WgMLST of  
148 ST-6460, representing the second most common ST, revealed 87-89 allelic differences between  
149 the western jackdaw isolates and a chicken isolate obtained in 2012 from the Finnish  
150 *Campylobacter* monitoring program (Fig. 2B). Western jackdaw ST-6460 isolates were more  
151 closely related to each other, as only 8 to 16 allelic differences occurred among the isolates,  
152 which had been collected from two different locations and on three separate occasions (Fig. 2B).  
153 *C. jejuni* pairs (CB294/CB286) and (CB329/CB358) in panel (B) were isolated on the same days  
154 and from the same sampling points, however originating from different towns.

155 WgMLST analysis of ST-2314 isolates from Finnish mallard ducks and human patients from the  
156 UK (isolated in 2010, 2011, and 2013) is shown in Fig. 2C. The isolates formed three clusters,  
157 differing by 195 to 303 alleles. Between the mallard duck ST-2314 isolates, only 2 to 7 allelic  
158 differences were detected, and 17 to 21 allelic differences (approximately 1% of the 1614 shared  
159 loci) were found between the five mallard duck isolates and a clinical isolate from the UK  
160 (OXC8492) in 2013 (Fig. 2C).

161 WgMLST of ST-19, describing the allelic differences between the *C. jejuni* isolates from  
162 pheasants and human patients, collected in the UK in 2010-2014  
163 ([www.pubMLST.org/campylobacter](http://www.pubMLST.org/campylobacter)) and in Finland in 2012 (19), is shown in Fig. 2D. The  
164 isolates formed four distinct (from 600 to 945 allelic differences) clusters. One of the clusters  
165 that consisted of all of the genetically highly related pheasant isolates (differing by 3 to 4 alleles)  
166 and one clinical isolate obtained from the UK in 2014 (OXC9006), which diverged from each  
167 other by 33 to 37 allelic differences, accounted for 2% of the 1581 shared loci (Fig. 2D).

168 **Description of genetic features**

169 ***Integrated elements (CJIEs)***

170 Integrated elements CJIE1, 2, 3, and 4 were present in 20% (17/87), 86% (77/87), 85% (74/87)  
171 and 14% (12/87) of the western jackdaw isolates, respectively. However, there were large  
172 deletions and other major differences in the genomic structure of CJIE2 in the majority of the  
173 isolates compared with reference strain RM1221 (20). Among mallard duck isolates, integrated  
174 elements CJIE1, 2, 3, and 4 were present in 3% (1/36), 39% (14/36), 81% (29/36), and 3% (1/36)  
175 of the isolates, respectively. The only integrated element present in pheasant isolates was CJIE4,  
176 which was present in 100% of the isolates.

177 ***Type VI secretion system***

178 A total of 43 of 87 (49%) of the western jackdaw isolates carried a type VI secretion system  
179 (T6SS), similar in gene synteny and sequence (96% query coverage and 96% identity in  
180 BLASTN) to the T6SS of strain 108 (21). Interestingly, the majority (72%) of the mallard duck  
181 isolates also carried the T6SS, whereas none of the pheasant isolates carried either the CJIE3 or  
182 T6SS.

183 ***Cytolethal distending toxin***

184 Fig. 3 shows an alignment of the cytolethal distending toxin (*cdtABC*) gene clusters of western  
185 jackdaw isolate CB287 against the reference strain NCTC 11168. The *cdtABC* operon, located in  
186 the same genomic site as in NCTC 11168, was complete in only two of the western jackdaw  
187 isolates, missing completely in three isolates, and degenerated and thus likely dysfunctional in  
188 most (82 of 87) of the western jackdaw isolates (e.g. CB287, Fig. 3). However, another  
189 homologous *cdtABC* gene cluster (69% identity, 84% query coverage against other *C. jejuni*

190 sequences in nr using BLASTN) was present in another location of the genome in 59% (51/87)  
191 of the western jackdaw isolates (e.g. CB287, Fig. 3). For further analysis, a phylogenetic  
192 neighbor-joining tree was constructed based on the conserved amino acid sequences of the *cdtB*  
193 gene. The novel jackdaw CdtB sequence was relatively distinct from those of other *C. jejuni*  
194 strains and even from other *Campylobacter* spp., but highly similar to those of crow *C. jejuni*  
195 isolates from California, USA (Fig. 4). The two western jackdaw isolates that had an intact  
196 *cdtABC* operon, located in a similar position as in strain NCTC 11168, represented ST-4565 and  
197 ST-7841, which were clearly distinct from the majority of the western jackdaw STs also in the  
198 ClonalFrame genealogy and located in the generalist clade (Fig. 1). Similarly, the CdtB  
199 phylogenetic tree (Fig. 4) showed that the sequences of these two isolates were more closely  
200 related to other *C. jejuni* strains, including the pheasant isolates from our study and generalist *C.*  
201 *jejuni* isolates from crows in a previous study (17).

202 The *cdtABC* operon was degenerated also in all mallard duck isolates, except one isolate that  
203 carried the novel *cdtABC* gene cluster, homologous to most of the western jackdaw isolates. This  
204 mallard duck isolate represented ST-6228, which was located in the same clade with western  
205 jackdaw STs in the ClonalFrame genealogy (Fig. 1) and had identical or closely related CdtB  
206 amino acid sequence to the western jackdaw and crow isolates (Fig. 4). In contrast to western  
207 jackdaw and mallard duck isolates, the *cdtABC* operon (similar to strain NCTC 11168) was  
208 intact and most likely functional among all pheasant isolates (ST-19).

### 209 ***Antimicrobial resistance-associated genetic markers***

210 The *tetO* gene encoding for tetracycline resistance ribosomal protection protein (TetO) was  
211 found to be present in 14% (12/87) of the jackdaw isolates, but only in one of the 36 mallard  
212 duck isolates (3%) and none of the pheasant isolates. The 23S rRNA gene in positions 2074 and

213 2075 was wildtype, suggesting susceptibility to macrolides. Gene *AadE*, conferring streptomycin  
214 resistance, was absent among all bird isolates. No mutations associated with ciprofloxacin  
215 resistance in the *gyrA* gene were detected among mallard ducks, but one T86I substitution in  
216 *gyrA* was present in a western jackdaw isolate. Similarly, the T86I mutation in *gyrA* was found  
217 among all pheasant isolates.

218 The neighbor-joining tree representing the alignment of family class D beta-lactamase (*bla-OXA*)  
219 gene sequences is shown in Supplementary Figure S2. All pheasant isolates carried *bla-OXA 61*  
220 family class D beta-lactamase gene with sequences identical to *C. jejuni* type strain NCTC  
221 11168. In contrast, *bla-OXA 184* family class D beta-lactamase was found to be present in 69%  
222 (60/87) of the western jackdaw isolates and in all mallard duck isolates. Furthermore, based on  
223 the beta-lactamase gene sequences among the western jackdaws and mallard ducks, they formed  
224 separate branches in the neighbor-joining tree (Fig. S2), with the exception of the sequences of  
225 two mallard duck isolates: SS6R (ST-6228) and SO-68 (ST-7843), located in the same cluster as  
226 the majority of the western jackdaw STs in the ClonalFrame analysis (Fig. 1).

## 227 **Discussion**

228 Birds are well-recognized natural reservoirs of *C. jejuni* and different wild bird species have  
229 been found to carry *C. jejuni*, among other *Campylobacter* spp., with variable frequencies. Thus,  
230 wild birds may pose a risk for public health either indirectly by transmitting *C. jejuni* to other  
231 animals, food production farms, and environmental waters, and through these, also to humans, or  
232 directly, when bird meat is consumed as game (4). This study is the first to characterize *C. jejuni*  
233 isolates from western jackdaws and game birds using MLST and comparative genomics in order  
234 to elucidate their potential public health significance. Western jackdaws have not been

235 previously investigated extensively. In this study, we found 43% (n=212) of our western jackdaw  
236 isolates to carry *C. jejuni*. In addition to western jackdaws, 76% (n=108) of the mallard ducks  
237 carried *C. jejuni*. An earlier study from New Zealand reported 23% (n=702) of wild mallard  
238 ducks to be positive for *C. jejuni* (5), and in a study from the UK, farmed mallard ducks carried  
239 high frequencies (93.3-100%) of *Campylobacter*, emphasizing their role also as a risk for human  
240 health (22). The relatively high frequency and diversity of *C. jejuni* among mallard ducks in this  
241 study may be at least partly a consequence of mixing with natural *C. jejuni* populations from  
242 other waterfowl since the farmed-mallard ducks were raised in natural ponds, which were co-  
243 habited by wild birds. In contrast to western jackdaws and mallard ducks, a low prevalence of *C.*  
244 *jejuni* was found among farmed pheasants (9%). In a previous study from the Czech Republic,  
245 70% of pheasants that were farmed with intensive production were positive for *Campylobacter*  
246 sp., 41% being *C. jejuni* (23). However, in the same study, a lower frequency (25.7%) was  
247 detected in wild pheasants; only 16% were *C. jejuni*-positive (23). Colonization dynamics of *C.*  
248 *jejuni* in wild birds is complicated and may depend on, for instance, host ecology, age of the  
249 bird, social behavior, and season (24).

250 Only limited epidemiological data exist on cases where western jackdaws, mallard ducks, or  
251 pheasants have been suspected as a source of human infections. However, from the 1980s to  
252 1990s in the UK, several milkborne outbreaks were associated with western jackdaws and  
253 magpies that were pecking milk bottles located outside the front doors of houses (25, 26). Also,  
254 in a Finnish epidemiological study in the 1990s we found two patients suspected of having  
255 acquired campylobacteriosis after contact with pheasants (27).

256 The high occurrence of novel or rarely detected STs, which have only infrequently previously  
257 been associated with human disease, suggests that while western jackdaws are not a major, they

258 are a potential source of campylobacteriosis in humans. The most commonly detected STs were  
259 ST-1282 and ST-6460, the former being previously isolated from a wild bird in Sweden and  
260 environmental water in Luxembourg ([www.pubMLST.org](http://www.pubMLST.org)). ST-6460, in turn, has been  
261 infrequently detected in a chicken slaughter batch in Finland in 2012 (2) and in a human patient  
262 in Sweden ([www.pubMLST.org](http://www.pubMLST.org)). Other STs found among western jackdaws isolated in human  
263 stools, included ST-1539 (UK and Netherlands), ST-6589 (Sweden), and ST-6590 (Sweden)  
264 ([www.pubMLST.org/campylobacter](http://www.pubMLST.org/campylobacter)). However, overall STs previously reported in human  
265 disease accounted for only 9 of 87 isolates (10%), and those reported previously in chickens (ST-  
266 6460 and ST-5543) accounted for 7 of 87 isolates (8%), supporting the result that jackdaws are  
267 not a major source of *C. jejuni* in human infections. Chicken farming is located in the same area  
268 in western Finland where the sampling of western jackdaws took place, suggesting that western  
269 jackdaw flocks living close to chicken farms may pose a risk of contamination.

270 Despite the high frequency (76%) of *C. jejuni* among Finnish mallard ducks, all STs differed  
271 from those previously found among Finnish human patients with infections acquired from  
272 domestic sources (18, 19, 28, 29). However, the five most common STs (ST-2314, ST-1299, ST-  
273 2839, ST-991, and ST-995) have previously been reported in the pubMLST database in different  
274 geographical areas from human stools (except ST-2839, unknown source) and from other wild  
275 birds (except ST-2314 and ST-2839), suggesting that these STs may have a wider host range and  
276 the capability of also causing human infections. Furthermore, the ST-2314 mallard duck isolates,  
277 representing one-third of all of the mallard duck isolates based on PFGE types (data not shown),  
278 differed in wgMLST analysis from each other by only 2 to 7 alleles, indicating that these isolates  
279 represent the same clone, which was also found to be genetically highly similar to a human  
280 isolate previously isolated in the UK. In addition, both the farmed and wild mallard ducks in this

281 study carried ST-995 (UA), which has been detected among mallard ducks also in Sweden (16)  
282 and New Zealand (5) (Fig. S3), suggesting a strong host association of this ST with mallard  
283 ducks.

284 All *C. jejuni* isolates from pheasants represented ST-19, which has previously been frequently  
285 isolated from human patients with gastroenteritis as well as from a wide variety of different hosts  
286 worldwide (22, 30-32). Since all the pheasants carried the same clone of *C. jejuni* (3 to 4 allelic  
287 differences in wgMLST analysis), but were hunted on three separate occasions and from two  
288 different locations, the birds had most likely been colonized already in the breeding farm at a  
289 very early stage, before they were moved to the separate hunting farms.

290 The phylogenomic tree based on the core genomes clearly indicated that although the western  
291 jackdaw isolates form a very distinct set of strains from the rest of the *C. jejuni* population, they  
292 do not form a cohesive single population. ClonalFrame genealogy further confirmed that the  
293 majority of the *C. jejuni* STs from western jackdaws clustered separately from the other STs  
294 previously detected in human patients, poultry, bovine, and other wild birds in Finland (2, 7, 18,  
295 19, 28, 29), confirming the suggestion that these STs have evolved in western jackdaws and are  
296 not frequently transferred between different animal host species. Only two STs (ST-7841 and  
297 ST-4565) were located in the same branch, which comprised also STs isolated from human  
298 patients or poultry. ClonalFrame also showed that most of the mallard duck STs formed two  
299 branches, containing also STs detected previously from Finnish barnacle geese (7), which may  
300 indicate that these STs have adapted more widely to waterfowl. This was supported by the  
301 phylogenomic analysis. In conclusion, ClonalFrame genealogy of our study showed concordance  
302 with earlier studies (6, 7, 16), revealing that wild birds are mostly colonized by host-adapted

303 STs, but also have a minor set of STs shared with other hosts, including domestic animals and  
304 human patients.

305 Comparative genomics using wgMLST was done to explore genomic diversity within the most  
306 frequently detected STs. In conclusion, wgMLST using GeP (14) to detect closely related *C.*  
307 *jejuni* isolates revealed that birds living in flocks in close contact are colonized by both  
308 genetically highly related (e.g. ST-6460, ST-2314, and ST-19) and diverse STs (e.g. ST-1282).  
309 Each strain and ST will most likely have their own capability to evolve, as shown earlier for ST-  
310 45 CC (12), and for ST-2314 and ST-19 in this study, but more spatial and temporal data from  
311 each genetic lineage are needed to make robust conclusions. Our results add to the evidence that  
312 comparative genomics within STs, using wgMLST, is a suitable method to analyse the genomic  
313 relationships of *C. jejuni* isolates. These results further confirm the results of our previous  
314 studies, where we found that isolates originating from the same outbreak usually have only a few  
315 allelic differences, whereas isolates from unassociated sources more likely have tens to several  
316 hundreds of allelic differences (33-35).

317 Even though several putative virulence factors of *C. jejuni* have been suggested to be associated  
318 with the pathogenesis of human gastroenteritis (20, 21, 36, 37), their functions are not well-  
319 known and it remains unclear whether all strains have the capability to cause human infections  
320 (38). Most of the STs detected among western jackdaw and mallard duck isolates were novel,  
321 thus they have not been associated previously with human infections or detected in other  
322 animals. Therefore, we studied genetic characteristics of these isolates to screen for virulence-  
323 associated genes in their genomes. One of the most recently described putative virulence-  
324 associated systems in *C. jejuni* (39) and in *C. coli* (40) is T6SS. In our study, a complete T6SS  
325 gene cluster was found to be common among western jackdaw (46%) and mallard duck isolates



326 (72%). The high frequency of T6SS in our study is an interesting result because T6SS has been  
327 recognized to contribute to bacterial pathogenesis by toxic effects on host cells or competing  
328 bacterial species (39). The *C. jejuni* T6SS has been found to have pleiotropic effects, ranging  
329 from adaptation to host cell adherence, *in vivo* colonization, invasion, and cytotoxicity towards  
330 human erythrocytes (39, 41, 42). In addition, patients harboring a bacterial strain with T6SS  
331 often had bacteremia (39), suggesting more severe disease. The T6SS gene cluster has been  
332 found with variable frequencies in different studies, most probably depending on the origin of  
333 the strain. Commonly, approximately 10% of studied strains harbor T6SS, containing 13 ORFs  
334 similar to those described in *C. jejuni* strain 108 (39). T6SS was not detected among common  
335 STs and clonal lineages occurring in human patients and animal sources in the UK (e.g. ST-45  
336 CC, ST-21 CC), but it was more often detected among human patient isolates from Vietnam and  
337 Pakistan, representing rare STs not commonly detected in Europe (42). This finding corroborates  
338 our results because most of the western jackdaw and mallard duck isolates had novel STs, which  
339 were not detected previously in human patients or other animal hosts. More studies on patients  
340 infected with uncommon STs containing T6SS are warranted to strengthen the evidence of an  
341 association between bacteremia and certain genetic lineages of *C. jejuni*.

342 Another virulence-associated system known to be common in *C. jejuni* is the cytolethal  
343 distending toxin (CDT), encoded by a three component operon *cdtABC* (37). CdtB is a DNase  
344 that causes DNA double-strand breaks in the nucleus, resulting in cell cycle arrest at the G2/M  
345 stage and apoptosis (43). Previous PCR-based studies directed at the CDT operon or a single  
346 gene of the operon have shown more than 95% of *C. jejuni* strains to be positive (36). In the  
347 present study, all, except two, of our western jackdaw isolates carried a *cdtABC* operon, but  
348 surprisingly it was degenerated in most (94%) of the isolates. The two isolates with an intact

349 copy of the *cdtABC* clustered more closely with the generalist clade in the ClonalFrame  
350 genealogy and also in the CdtB phylogeny. In addition, the gene cluster was totally missing from  
351 three of the jackdaw isolates. Interestingly, almost all isolates with a degenerated *cdtABC* locus  
352 also had another intact homologous *cdtABC* gene cluster. The newly identified *cdtABC* gene  
353 cluster was consistently present only in the western jackdaw clade in the ClonalFrame  
354 genealogy. This is the first time, to our knowledge, that another complete *cdtABC* gene cluster  
355 has been reported to be present in *C. jejuni*. Further analysis of the CdtB sequences of the newly  
356 identified gene cluster revealed that the sequences of *C. jejuni* isolates from western jackdaws  
357 were identical or closely related to those of crow isolates collected in California, USA (17).  
358 Comparison of the CdtB sequences also revealed that the jackdaw and crow sequences were  
359 more closely related to *C. lari* than other *C. jejuni* isolates, including the pheasant isolates from  
360 this study. This is an interesting finding and warrants further studies. All mallard duck isolates  
361 carried only the degenerated *cdtABC* locus. Whether the degeneration of the *cdtABC* locus is  
362 connected only to wild bird-associated genetic lineages remains unanswered and awaits future  
363 research.

364 The *tetO* gene, predicting resistance against tetracycline (TET), was found to be present among  
365 14% of the western jackdaw isolates. Among mallard ducks, the *tetO* gene was present in only  
366 one isolate, and in none of the pheasant isolates, suggesting low resistance against TET. The  
367 T86I mutation in *gyrA*, previously linked to high fluoroquinolone-resistance in *Campylobacter*  
368 strains (44, 45), was found in only one jackdaw isolate. Among mallard ducks, *gyrA* was found  
369 to be wildtype, however, among pheasants, the T86I substitution in *gyrA* was found in all  
370 isolates, predicting resistance against ciprofloxacin. The MIC value of ciprofloxacin was  
371 confirmed to be  $\geq 16$   $\mu\text{g/ml}$  and 64  $\mu\text{g/ml}$  for nalidixic acid using VetMIC Camp EU (SVA,

372 Sverige). In a previous study, no resistance against ciprofloxacin (CIP) was found in wild bird  
373 isolates in Finland (46), indicating that the results of the present study are mostly in line with the  
374 low occurrence of fluoroquinolone resistance among wild birds since the pheasant isolates most  
375 likely represented a single clone. It is intriguing why this single clone was so successful and no  
376 other types were found among the pheasant isolates. The farm raising the pheasants for hunting  
377 did not use any antibiotics and this may be excluded as a reason for the clonal expansion of this  
378 resistant type. Contact with wildlife is likely in this production system from very early stages on,  
379 however, only after the birds are released to the surroundings as 3-4 week old. Interestingly, a  
380 small proportion of the hatched pheasant eggs are imported from France on a yearly basis, and  
381 this may be a possible source of resistance since the birds carried the same clone on separate  
382 farms even though they had been separated as one-day-old chicks. Furthermore, ST-19 is a  
383 common generalist type known to colonize a variety of hosts, including wild bird in Canada and  
384 ducks in the UK (<https://pubmlst.org/campylobacter/>), and carry the CIP-NAL resistance  
385 phenotype (47, 48). According to EFSA and ECDC, resistance against ciprofloxacin is generally  
386 high among human, chicken and turkey *C. jejuni* isolates in the EU, and showing a rising trend  
387 (49). Further studies will reveal if fluoroquinolone resistance is on the rise also among *C. jejuni*  
388 in wild bird populations. A previous study (50) reported that certain strains with this mutation in  
389 *gyrA* showed no biological cost and fluoroquinolone-resistant strains could even outcompete  
390 susceptible ones *in vivo* in chickens in the absence of selective pressure, which could also  
391 explain the clonal expansion of successful clones. Genomic markers, conferring streptomycin  
392 (*AadE*) and macrolide (23S rRNA gene mutation in positions 2074 and 2075) resistance, were  
393 absent among all bird isolates. Overall, antimicrobial resistance has remained low in Finland

394 among the *C. jejuni* isolates of domestic origin relative to several other countries (46) and seems  
395 to be low also in birds migrating to Finland.

396  $\beta$ -lactams (including penicillin, amoxicillin, and ampicillin) have been widely used in veterinary  
397 and clinical medicine, however, emerging resistance has compromised their use. High-level  $\beta$ -  
398 lactam resistance and  $\beta$ -lactamase activity have been shown to be linked to an upregulated *bla*-  
399 *OXA 61* gene in *C. jejuni* (51). Interestingly, among our *C. jejuni* collection the *bla-OXA 184*  
400 family (instead of *bla-OXA 61*) class D beta-lactamase was present in 69% of the western  
401 jackdaw isolates, and it was also present in all mallard duck isolates. In contrast, all pheasant  
402 isolates carried the OXA-61 family class D beta-lactamase. Further phylogenetic analysis  
403 revealed that the *bla-OXA 184* sequences between mallard duck and western jackdaw isolates  
404 differed markedly, suggesting an evolutionary link with the host. Previously, the OXA 184  
405 family beta-lactamases have been found to be associated also with crow isolates compared with  
406 OXA 61, which has been associated with strains isolated from either domestic animals or human  
407 patients (17). These results reveal that OXA 184 family class D beta-lactamase is for an  
408 unknown reason associated with wild bird isolates, but future studies will extend our knowledge  
409 of their evolution and functionality.

410 In conclusion, phylogenetic studies performed on MLST data, CdtB and beta-lactamase gene  
411 sequences support a unique evolutionary history of *C. jejuni* in western jackdaws. Due to a high  
412 frequency of novel STs and STs only rarely detected among human patients and broiler batches,  
413 western jackdaws are, however, considered to be an infrequent source of campylobacteriosis in  
414 humans and contamination source of poultry production. Although the STs among western  
415 jackdaws and mallard ducks seem to be clearly host-associated, they may have the capability to  
416 transfer into domestic animals as well as occasionally cause human infections. Game birds

417 especially may pose a higher risk for acquiring campylobacteriosis, and both the carcasses and  
418 meat should be handled accordingly. More evidence is anticipated to accumulate when MLST,  
419 particularly wgMLST, is applied more extensively on different sources worldwide.

## 420 **Materials and methods**

### 421 **Bacterial isolates**

422 *Campylobacter* spp. was isolated from western jackdaws from two small towns, located in  
423 Southern Finland (Lahti) and Western Finland (Seinäjoki), during a five-month period from  
424 September 2014 to February 2015. Fecal droppings (n=212) were collected by placing a plastic  
425 carpet under the known roosting trees of jackdaws. When jackdaws had settled in their roosting  
426 trees, the flock was scared away to cause an automatic defecation of the birds. Individual  
427 droppings were immediately swabbed into transport tubes (TS0001, Oxoid, Thermo Fisher  
428 Scientific, Vantaa, Finland) and delivered to the laboratory chilled (+4°C). Contamination of the  
429 collected fecal material is unlikely as we checked that no other bird species were present in the  
430 same tree before scaring the jackdaw flocks away. Of note, especially hooded crows tend to roost  
431 with jackdaws.

432 Samples from game birds that were farmed for hunting were collected during the hunting season  
433 in Southern Finland. The birds were shot by licensed hunters and no animals were killed for the  
434 purpose of this study. Mallard ducks (n=100) were hunted from September to November in 2014  
435 and pheasants (n=100) from November to December in 2013. The birds were plucked (mallard  
436 ducks) or skinned (pheasants) and eviscerated immediately after hunting. Intestinal samples were  
437 collected during evisceration, and transported to the laboratory chilled (+4°C). The mallard ducks  
438 were hunted on the surroundings of a single farm. The birds were hatched on another breeding

439 farm and three- to four-week-old birds were transferred and released to the natural ponds of the  
440 hunting farm where they were raised by regular feeding. Pheasants were hatched on the same  
441 farm as mallard ducks were raised on. Six batches stayed on the same farm for hunting and three  
442 batches of one-day-old chicks were transferred to yet another hunting farm where they were  
443 raised. In both hunting farms, the four-week-old birds were released into the surroundings where  
444 their feeding was continued.

445 In addition to farmed game mallard ducks, fresh fecal samples (n=8) from the wild mallard duck  
446 population, living in a lake of an urban park in Southern Finland, were collected from individual  
447 droppings into transport tubes (TS0001, Oxoid, Thermo Fisher Scientific, Vantaa, Finland) in  
448 November 2014.

449 Fecal samples were cultivated on mCCDA plates (Oxoid Ltd., Hampshire, UK). Typical colonies  
450 were Gram-stained and genomic DNA was extracted using PureLink™ Genomic DNA minikit  
451 (Invitrogen, Waltham, MA, USA). *Campylobacter* species was identified by species-specific  
452 PCR (52). DNA of all *C. jejuni* isolates from western jackdaws, pheasants, and wild mallard  
453 ducks was subjected to WGS.

454 For *C. jejuni*-positive game mallard duck isolates, pulsed-field gel electrophoresis (PFGE), using  
455 KpnI restriction enzyme, was first used to screen the diversity of PFGE types among the isolates.  
456 PFGE was performed as described previously (53). Thirty-five isolates, representing all of the  
457 different PFGE profiles (some in duplicate or triplicate), were selected and their DNA was  
458 subjected to WGS.

## 459 **Whole-genome sequencing**

460 Whole-genome sequencing (WGS) was performed using Illumina HiSeq Technology at the  
461 Institute for Molecular Medicine Finland (FIMM). Raw data were assembled into contigs using  
462 INNUca pipeline (<https://github.com/B-UMMI/INNUca>) and genome sequences (n=6) that did  
463 not pass the quality threshold were excluded from further analyses.

## 464 **Phylogenetic analyses**

465 A phylogenomic tree was constructed based on the core genomes of 1261 *C. jejuni* strains  
466 selected from the INNUENDO *Campylobacter jejuni* dataset (54) and including the genomes  
467 sequenced in this study (Supplementary Dataset S1). The genomes were annotated using Prokka  
468 1.12 (55). A pan genome analysis was performed using Roary 3.7.0 (56) with 95% protein  
469 identity as cutoff for defining gene clusters and the core genes. FastTree 2.1 (57) was used with  
470 the Jukes-Cantor model of nucleotide evolution for building the approximation of a maximum-  
471 likelihood (ML) phylogenetic tree based on the core genome alignment (99% shared loci in  
472 Roary analysis, including 942 core genes). When necessary the jobs were run in parallel using  
473 GNU Parallel (58). iTOL (59) v4.2.3 was used for visualization. The tree was rooted at midpoint  
474 for better visualization.

475 ClonalFrame genealogy was used to describe the phylogeny of the *C. jejuni* MLST allele  
476 sequences collected in this study and including also MLST sequence data of *C. jejuni* isolates  
477 from our previous studies originating from several sources (poultry, bovine, barnacle geese and  
478 human) in Finland (2, 7, 18, 19, 28, 29). ClonalFrame was run using default parameters (60), and  
479 the consensus tree was displayed in MEGA6 and labelled using CoreIDRAW X8.

480 GoeBURST full Minimum spanning tree (MST) was constructed using PHYLOViZ (61).  
481 Besides our wild bird isolates, MLST data for mallard ducks from Sweden (16) and New  
482 Zealand (5) were obtained from the PubMLST database ([www.pubMLST.org/campylobacter](http://www.pubMLST.org/campylobacter)).

### 483 **Comparative genomics**

484 Genome profiler (GeP) (14) was used in wgMLST to further compare the draft genomes. In  
485 addition to our isolates, WGS data from clinical isolates from the UK were obtained from the  
486 PubMLST database ([pubMLST.org/campylobacter](http://pubMLST.org/campylobacter)) for reference. The clinical ST-19 isolates  
487 from the UK were chosen to represent all available subtypes as defined by using coregenome  
488 (cg) MLST for 700 loci ([https://github.com/mickaelsilva/chewBBACA\\_deprecated/wiki](https://github.com/mickaelsilva/chewBBACA_deprecated/wiki)), and  
489 different years. Allelic differences between strains of each ST, obtained using GeP, were  
490 visualized using SplitsTree4 (62) and edited in CoreIDRAW X8.

491 RAST (Rapid Annotation using Subsystem Technology) server (63) was used for preliminary  
492 annotation and genomic characterization of the isolates. Sequence-based comparisons, genome  
493 browsing, and alignments for visualization of synteny of homologous genomic regions were  
494 performed using the SEED viewer (64, 65) with default parameters. Novel open reading frames,  
495 genes associated with cytolethal distending toxin (*cdtABC*) operon, integrated elements (CJIE1,  
496 2, 3, and 4), type VI secretion system (T6SS), family class D beta-lactamases, and antimicrobial  
497 resistance were further analyzed using the InterProScan (66) and by BLASTN and/or BLASTP  
498 searches against the non-redundant nucleotide/protein sequences database (nr) in GenBank.

499 Phylogenetic analyses of genes of interest were performed by aligning the amino acid sequences  
500 of the *cdtB* gene and nucleotide sequences of family class D beta-lactamases using MUSCLE  
501 (<https://www.ebi.ac.uk/Tools/msa/muscle/>) and MEGA6 (67) was used to construct a



502 phylogenetic neighbor-joining tree based on the alignments. The resulting phylogenetic trees  
503 were text edited in CorelDRAW 2017.

#### 504 **Sequence deposition**

505 Whole-genome sequences of those bird isolates that represented novel STs were deposited to the  
506 pubMLST database ([www.pubMLST.org/campylobacter](http://www.pubMLST.org/campylobacter)) to assign new sequence types and  
507 allelic profiles.

508 The whole-genome sequences of all of the isolates sequenced in this study have been deposited  
509 in GenBank under BioProject PRJNA430314. The relevant BioSample and sequence accession  
510 numbers are listed in Supplementary Table S1. NCBI Prokaryotic Genomes Annotation Pipeline  
511 (PGAP) was used to annotate the genomes.

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## 753 **Figure legends**

754 Figure 1. ClonalFrame genealogy of MLST allele sequences in different hosts detected in this  
755 study (western jackdaws and mallard ducks) and previously in Finland (2, 7, 18, 19, 28, 29).  
756 Different hosts and combinations of hosts are indicated with different colors, and those STs that  
757 were further analyzed in wgMLST are indicated with arrows. A dashed line separates the STs  
758 detected from human patients, poultry, bovine, and wild birds (generalist clade) from the STs  
759 mainly detected from western jackdaws (western jackdaw clade).

760 Figure 2. WgMLST describing the allelic differences among shared loci of ST-1282 (A), ST-  
761 6460 (B), ST-2314 (C) and ST-19 (D) isolates using GeP (14). Numbers between strains indicate  
762 allelic differences. Jackdaw isolates are indicated in dark blue, chicken isolate in light blue,  
763 mallard duck isolates in orange, pheasant isolates in red, clinical isolates from the UK  
764 ([www.pubMLST.org/campylobacter](http://www.pubMLST.org/campylobacter)) in black and Finland in green.

765 Figure 3. Seed Viewer homology-based sequence alignment of the two *cdtABC* gene clusters  
766 (top and bottom rows) of western jackdaw strain CB287. The chromosomal region of the focus  
767 gene (top) is compared with four similar organisms (*C.* = *Campylobacter*, *H.* = *Helicobacter*).  
768 The graphic is centered on the focus gene (*cdtC*), which is red and numbered 1. Sets of genes  
769 with similar sequence are grouped with matching number and color code. Genes that are not  
770 conserved remain unnumbered and are shown in gray.

771 Figure 4. Neighbor-joining tree representing the alignment of CdtB amino acid sequences.  
772 Accession number, species, and strain number are indicated for each sequence. Strains from this  
773 study are indicated in boldface and with CB for western jackdaw, SO or SS for mallard duck,  
774 and FA for pheasant in the strain name.