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Inconsistency of Phenotypic and Genomic Characteristics of *Campylobacter fetus* Subspecies Requires Reevaluation of Current Diagnostics

Linda van der Graaf-van Bloois,[a,b] William G. Miller,[c] Emma Yee,[c] Martine Rijnsburger,[d] Jaap A. Wagenaar,[a,b,e] Birgitta Duim[a,b]

Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands[a]; WHO Collaborating Centre for Campylobacter and OIE Reference Laboratory for Campylobacteriosis, Utrecht, the Netherlands[b]; Produce Safety and Microbiology Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Albany, California, USA[c]; VU University Medical Center, Department of Medical Microbiology and Infection Control, Amsterdam, the Netherlands[d]; Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands[e]

Classifications of the *Campylobacter fetus* subspecies *fetus* and *venerealis* were first described in 1959 and were based on the source of isolation (intestinal versus genital) and the ability of the strains to proliferate in the genital tract of cows. Two phenotypic assays (1% glycine tolerance and H_2S production) were described to differentiate the subspecies. Multiple molecular assays have been applied to differentiate the *C. fetus* subspecies, but none of these tests is consistent with the phenotypic identification methods. In this study, we defined the core genome and accessory genes of *C. fetus*, which are based on the closed genomes of five *C. fetus* strains. Phylogenetic analysis of the core genomes of 23 *C. fetus* strains of the two subspecies showed a division into two clusters. The phylogenetic core genome clusters were not consistent with the phenotypic classifications of the *C. fetus* subspecies. However, they were consistent with the molecular characteristics of the strains, which were determined by multilocus sequence typing, *sap* typing, and the presence/absence of insertion sequences and a type I restriction modification system. The similarity of the genome characteristics of three of the phenotypically defined *C. fetus* subsp. *fetus* strains to *C. fetus* subsp. *venerealis* strains, when considering the core genome and accessory genes, requires a critical evaluation of the clinical relevance of *C. fetus* subspecies identification by phenotypic assays.

Campylobacter fetus is an important veterinary pathogen and is associated with genital infections in cattle and sheep that result in abortion and infertility (1). Based on clinical and phenotypic observations, the species *C. fetus* was subdivided into two subspecies, *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* (2). *C. fetus* subsp. *venerealis* was of venereal origin, had a strong ability to cause abortions, and persisted in the genital tracts of cattle and sheep, whereas *C. fetus* subsp. *fetus* was of intestinal origin, caused only sporadic abortions, and was cleared from the genital tract of the cow following the abortion. The two subspecies were phenotypically differentiated with the 1% glycine tolerance and H_2S production tests; *C. fetus* subsp. *fetus* is positive in both tests (it is glycine tolerant and produces H_2S), and *C. fetus* subsp. *venerealis* is negative in both tests (it does not grow in the presence of 1% glycine and does not produce H_2S) (2). *C. fetus* strains that established themselves in the genital tract of a nonpregnant cow, like the venereal *C. fetus* subsp. *venerealis* strains, were isolated (3); however, the glycine resistance of these strains was reduced compared to that of most *C. fetus* subsp. *fetus* strains, and they were positive in the H_2S test, like the intestinal *C. fetus* subsp. *fetus* strains (4). They were classified as an intermediate group (4) and designated *Campylobacter fetus* subsp. *venerealis* bv. Intermedius (1).

For molecular subspecies identification, several PCR assays have been described, but they lack specificity (5). The subspecies can be genetically differentiated with multilocus sequence typing (MLST) (6) and amplified fragment length polymorphism (AFLP) analyses (7), but these methods are laborious and therefore not useful for routine diagnostic methods. The rationale behind the differentiation between the *C. fetus* subspecies is the supposed difference in pathogenicity and disease epidemiology. *C. fetus* subsp. *venerealis* is described as the causative agent of bovine genital campylobacteriosis (BGC), a statutory disease in many countries of the world and listed by the World Organization for Animal Health (OIE); in contrast, *C. fetus* subsp. *fetus* is associated with sporadic abortions (8).

Comparative genomics of two *C. fetus* strains revealed several unique regions for both subspecies, as shown by Kienesberger et al. (9); *C. fetus* subsp. *venerealis* contained multiple unique regions representing insertion sequences and genomic islands with type IV secretion system components and phage-related/hypothetical proteins (9), and *C. fetus* subsp. *fetus* contained clustered regularly interspaced short palindromic repeat (CRISPR)-*cas* loci and unique genes involved in lipopolysaccharide (LPS) biosynthesis (9). These data suggest that *C. fetus* subspecies can be distinguished on genomic features, but comparative genomics of a larger set of *C. fetus* strains is lacking. The aim of this study is to characterize *C. fetus* strains of both subspecies based on whole-genome sequencing data and to compare the results of classification based on core and accessory genome analysis with the current *C. fetus* subspecies identification based on phenotypic assays.

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Address correspondence to Birgitta Duim, b.duim@uu.nl.

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MATERIALS AND METHODS

Bacterial strains: phenotyping and genotyping. *C. fetus* strains (Table 1) were grown on heart infusion agar supplemented with 5% sheep blood (BioTrading, Mijdrecht, the Netherlands) for 2 days under microaerobic conditions (6% O_2, 7% CO_2, 7% H_2, 80% N_2, [Anoxomat; Mart Microbiology, Lichtenvoorde, the Netherlands]). The subspecies of the strains were phenotypically identified with the 1% glycine tolerance test and with a test of hydrogen sulfide (H_2S) production in a medium with 0.02% cysteine-hydrochloride, as described previously (1). Molecular identification was performed with MLST (6) and AFLP analysis (7).

Whole-genome sequencing. Whole-genome sequence data of 21 *C. fetus* strains were obtained using Roche GS-FLX Titanium sequencing. Roche 454 reads were assembled into contigs using the Newbler assembler (v2.6). The genomes of two *C. fetus* strains, 04/554 and 97/608, were closed through assembly of the Roche 454 contigs into scaffolds by using Perl scripts. To validate the assembly of the contigs and to determine the orientations and order of the scaffolds, a circular high-resolution AflII restriction map of the genome was generated by optical mapping (Argus Optical Mapper; OpGen, Inc., Gaithersburg, MD). The assembly of the *sap* locus, genomic islands, regions with insertion sequences, and repeats was confirmed with PacBio Continuous Long Reads (Keygene N.V., Wageningen, the Netherlands). All base calls and polymeric tracts were validated using the high-depth Illumina MiSeq reads. The genomes of strains 04/554 and 97/608 were annotated as described previously (10).

Phylogenetic analysis of core and accessory genomes. Three available closed *C. fetus* genomes were used as references: strain 82-40 (GenBank accession number CP000487), strain 84-112 (GenBank accession numbers HG004426 and HG004427), and strain 03/293 (GenBank accession numbers CP006999 to CP007002). The amino acid sequences of the open reading frames (ORFs) encoded by five genomes (the three reference genomes plus two genomes [04/554 and 97/608] sequenced in this study) were used as input for an all-versus-all sequence similarity search using BLASTp ($-$e 0.0001, >80% similarity cutoff). ORFs that exist in each of the five strains (>80% identity over at least 80% of the protein length) were considered to be part of the *C. fetus* core genome. The ORF sequences of strain 82-40 were used as reference sequences of the core genes. Regions encoding the *sap* locus, genomic islands, restriction modification (RM) systems, prophages, and insertion sequences were considered accessory genes.

The accessory genes in the Roche 454 contigs of 21 *C. fetus* strains were identified with a local BLASTn analysis ($-$e 0.0001, >80% similarity cutoff) against the identified accessory genes of the five closed *C. fetus* strains. The strains were considered positive for the specific accessory regions if the BLASTn match was >80% over at least 80% of the region.

The phylogenetic analysis of the core genomes was performed as follows. The nucleotide sequences of the predicted genes of the Roche 454 contigs were generated using GeneMark v2.8 (11). For each core gene, the corresponding nucleotide sequence of each strain was extracted and aligned on a gene-by-gene basis using MUSCLE (12). The alignments were concatenated into a contiguous sequence for each *C. fetus* strain. From this concatenated alignment, a phylogenetic maximum-likelihood tree was built using RAxML v7.2.8 under the GTRCAT model.

Nucleotide sequence accession numbers. The genome sequence of *C. fetus* subsp. *fetus* strain 04/554 has been deposited in GenBank under the accession numbers CP008808 and CP008809, and the sequence of *C. fetus* subsp. *venerealis* strain 97/608 has been deposited under the accession numbers CP008810 to CP008812.

RESULTS

Genome features. The genome of *C. fetus* subsp. *fetus* strain 04/554 is a circular chromosome of 1,800,764 bp with an average G+C content of 33.2% and one megaplasmid of 25,862 bp. The genome of *C. fetus* subsp. *venerealis* strain 97/608 has a circular chromosome of 1,935,028 bp with an average G+C content of 33.3% and contains two megaplasmids of 38,272 bp and 27,124 bp. The general features of the assembled genomes are shown in Table 2. The genome features of strains 03/293, 82-40, and 84-112 have been described previously (9, 10) and are summarized in Table 2.

Phylogeny of the *C. fetus* core genome. A comparison of five closed *C. fetus* genomes (of strains 82-40, 84-112, 04/554, 97/608, and 03/293) revealed highly syntenic genomes, which shared >90% sequence identity. The core genome of *C. fetus* was defined on the ORFs present in the closed genomes of five *C. fetus* strains and consisted of 1,409,454 bp and 1,509 ORFs. The core genome was then identified in an additional 18 *C. fetus* strains, and the genetic distances of all 23 core genomes were visualized with a phylogenetic maximum-likelihood tree (Fig. 1). The phylogenetic tree is arranged in two clusters of strains, designated clusters A and B. The majority ($n = 18$) of the strains were located in cluster A, whereas cluster B consisted of five strains with greater genetic distances.

Accessory genes of *C. fetus* strains. Major differences among the five closed *C. fetus* genomes were found in the accessory genes. Table 1 shows the identified genes belonging to the *sap* loci, insertion sequences, genomic islands, type I restriction-modification systems, prophages, and CRISPR-*cas* systems of the 23 analyzed *C. fetus* strains.

***sap* locus.** All analyzed *C. fetus* strains contained a *sap* locus, but the composition of this region differed between strains. *sap* type B strains belonged to the core genome cluster B, whereas *sap* type A strains were found in genome clusters A and B (Table 1). The *sap* loci of strains 84-112 and 97/608 contained transposable elements and a set of phage-related genes and hypothetical genes, indicating the presence of a prophage. These prophage sequences shared 100% identity but were inserted at different positions in the *sap* locus.

Genomic islands. Two genomic islands (GIs) that encoded a type IV secretion system (T4SS), as defined by Kienesberger et al. (9, 13), were identified in the chromosomes of the closed *C. fetus* genomes. These two chromosomally located T4SS regions were present in strains that were distributed over the two core genome clusters (Table 1).

Insertion sequences. Insertion sequences (ISs) were found in the chromosomes as well as in plasmids of the closed genomes of strains 84-112, 97/608, and 03/293. The identified ISs belonged to the IS*605*, IS*607*, and IS*200* families. The ISs were found only in the *C. fetus* strains belonging to core genome cluster A (Table 2). Each IS-positive strain contained all of the identified IS families.

Restriction modification system. Three of the closed *C. fetus* genomes, of strains 84-112, 97/608, and 03/293, contained a type I restriction modification (RM) system. This type I RM system consists of *hsd* genes with intervening ORFs, similar to the type I RM systems described for *C. jejuni* (14). The complete type I RM system is found only in *C. fetus* strains belonging to core genome cluster A (Table 2). The three other *C. fetus* strains of *sap* type A, 82-40, 110800-21-2, and BT 10/98, contained a remnant of this type, as the type I RM system of these strains lacked the *hsdS2* gene. The genomes of the *sap* type B strains did not contain any type I RM-encoding genes.

CRISPR-*cas* system. CRISPRs were present in all *C. fetus* strains, but only two *C. fetus* subsp. *fetus* strains, 82-40 and 98/v445, contained *cas* genes. These strains were not linked with the same core genome cluster.

Core genome clusters compared with accessory genes. The

TABLE 1 General characteristics of *C. fetus* strains

Strain	Country[a]	Source	Phenotype[b]			Genotype[c]		Core genome cluster	Accessory genes[d,e]					
			1% glycine tolerance	H_2S production	Phenotypic ID	AFLP	MLST (ST)		*sap* type	Prophage in *sap* locus	GI with T4SS	IS	Type I RM system	CRISPR-*cas*
82-40	USA	Human	+	+	Cff	Cff	6	B	A	−	−	−	+/−	+
110800-21-2	NL	Bovine (bull)	+	+	Cff	Cff	2	B	A	−	+	−	+/−	−
BT 10/98	UK	Ovine	+	+	Cff	Cff	2	B	A	−	−	−	+/−	−
04/554	AR	Bovine (fetus)	+	+	Cff	Cff	5	B	B	−	−	−	−	−
98/v445	UK	Bovine (bull)	+	+	Cff	Cff	3	B	B	−	+	−	−	+
03/293	AR	Bovine (fetus)	+	+	Cff	Cfvi	4	A	A	−	+	+	+	−
ADRI 1362	AR	Bovine	+	+	Cff	Cfvi	4	A	A	+	+	+	+	−
Zaf 65	SA	Bovine	+	+	Cff	Cfvi	4	A	A	−	−	+	+	−
01/165	AR	Bovine (mucus)	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
02/298	AR	Bovine (fetus)	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
03/596	AR	Bovine (fetus)	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
92/203	AR	Bovine (placenta)	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
97/532	AR	Bovine (mucus)	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
98/25	AR	Bovine (fetus)	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
WBT 011/09	UK	unknown	−	+	Cfvi	Cfvi	4	A	A	−	+	+	+	−
Zaf 3	SA	Bovine (fetus)	−	+	Cfvi	Cfvi	4	A	A	−	−	+	+	−
ADRI 513	AU	unknown	−	+	Cfvi	Cfv	4	A	A	−	−	+	+	−
CCUG 33872	CZ	unknown	−	+	Cfvi	Cfv	4	A	A	+	−	+	+	−
84-112	USA	Bovine	−	−	Cfv	Cfv	4	A	A	+	+	+	+	−
97/608	AR	Bovine (placenta)	−	−	Cfv	Cfv	4	A	A	+	+	+	+	−
B10	USA	Bovine	−	−	Cfv	Cfv	4	A	A	+	+	+	+	−
CCUG 33900	F	Bovine (abortion)	−	−	Cfv	Cfv	4	A	A	+	+	+	+	−
LMG 6570	BE	Bovine	−	−	Cfv	Cfv	4	A	A	+	+	+	+	−

[a] AR, Argentina; AU, Australia; BE, Belgium; CZ, Czech Republic; F, France; NL, the Netherlands; SA, South Africa; UK, United Kingdom.

[b] +, positive in assay; −, negative; Cff, *C. fetus* subsp. *fetus*; Cfv, *C. fetus* subsp. *venerealis*; Cfvi, *C. fetus* subsp. *venerealis* bv. Intermedius.

[c] MLST (ST), multilocus sequence typing (sequence type).

[d] GI with T4SS, genomic island with type IV secretion system; IS, insertion sequence; RM, restriction modification; CRISPR, clustered regularly interspaced short palindromic repeats.

[e] +, genes are present; −, genes are absent.

TABLE 2 Features of assembled *C. fetus* genomes

Feature	Data for strain (reference or source):				
	04/554 (this study)	97/608 (this study)	03/293 (10)	82-40 (9)[a]	84-112 (9)[a]
Genome size (bp)	1,800,764	1,935,028	1,866,009	1,773,615	1,926,886
G+C content (%)	33.2	33.3	33.3	33.3	33.3
No. of rRNA genes	3	3	3	3	3
No. of tRNA genes	43	43	43	43	43
No. of homopolymeric G+C tracts (>8 bp)	29	24	31	30	34
No. of open reading frames (no. of pseudogenes)	1,684 (68)	1,879 (60)	1,773 (48)	1,769	1,992
Plasmids	1	2	3	0	1
Size (bp)	25,862	38,272/27,124	91,400/35,326/3,993		61,141
G+C content (%)	29.0	31.3/28.1	29.4/33.0/31.4		31.5
sap locus type	B	A	A	A	A
Insertion elements (no. of copies)	0	14	13	0	5
Restriction or modification locus type	None	I	I	None	I
CRISPR-*cas* system	No	No	No	Yes	No

[a] With modifications from original publication.

presence of specific components of the accessory genes encoding prophages, genomic islands, and the CRISPR-*cas* system was not associated with a specific core genome cluster. The IS elements and complete type I RM system were exclusively found in the strains of cluster A. Strains of cluster B did not contain IS elements or a complete type I RM system, and they have different *sap* types.

Core genome clusters compared to subspecies identification. The subspecies were not consistently subdivided phenotypically or genotypically (Table 1). The results of the genotypic method MLST were consistent with those of the obtained core genome clustering; strains of cluster A all were MLST sequence type 4 (ST4), whereas cluster B consisted of strains with other MLST STs. Cluster B included two strains with similar MLST ST2s, and these strains had lower genetic distances than the other strains in cluster B. AFLP distinguished the strains within cluster A with a minor difference in fingerprints as *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. Intermedius (6), but this discrimination was not observed with the phylogenetic analysis of the core genomes. Strains that were phenotypically classified as *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. Intermedius belonged to core genome cluster A, but the eight phenotypically classified *C. fetus* subsp. *fetus* strains were dispersed among both genome clusters. This is represented in Fig. 1, in which all phenotypically identified *C. fetus* subsp. *fetus* strains are marked with an asterisk.

DISCUSSION

The original classification of the *C. fetus* subspecies is based on differences in the colonization of different niches and phenotypic characteristics (2–4). The two *C. fetus* subspecies are highly syntenic, sharing 92.9% sequence identity (9), and the subspecies cannot be distinguished by DNA-DNA hybridization (15), which questions the validity of subspecies differentiation and hampers an adequate taxonomic positioning of the subspecies. Furthermore, the reliability of the 1% glycine tolerance test can be influenced by the transduction of glycine tolerance by phages (16). Several molecular assays for the identification of the *C. fetus* subspecies have been published (5); however, none of the molecular assays corresponded fully to the phenotypic identification of the *C. fetus* subspecies (5, 6).

In this study, phylogenetic analysis of the core genomes subdivided the *C. fetus* strains into two clusters. All strains that were phenotypically identified as *C. fetus* subsp. *venerealis* (including *C. fetus* subsp. *venerealis* bv. Intermedius) clustered in one core genome cluster, contained only strains with MLST ST4, and harbored IS elements and a type I RM system. The strains phenotypically identified as *C. fetus* subsp. *fetus* were assigned to both clusters. Three *C. fetus* subsp. *fetus* strains, 03/293, Zaf 65, and ADRI 1362, were assigned to the core genome cluster with *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. Intermedius strains, despite their phenotypic identification of *C. fetus* subsp. *fetus*. The similarity of the MLST identification and core genome clusters can be explained by the fact that MLST is a small-scale reflection of the core genome. The phylogenetic analysis showed an obvious resemblance to the MLST STs of the strains; the genetic distances between strains with the same STs were very low, as shown in strains of ST4 and ST2, and the genetic distances increased for strains with different STs.

Campylobacter fetus subsp. *venerealis* bv. Intermedius is described as a phenotypic variant of *C. fetus* subsp. *venerealis* (1). The phenotypically identified *C. fetus* subsp. *venerealis* bv. Intermedius strains are all positioned together with *C. fetus* subsp. *venerealis* strains in cluster A of the phylogenetic tree. The accessory genes of *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. Intermedius strains showed no consistent presence of a *C. fetus* subsp. *venerealis*- or *C. fetus* subsp. *venerealis* bv. Intermedius-specific region. However, it is remarkable that, except for strain ADRI 513, all of the analyses with AFLP identified *C. fetus* subsp. *venerealis* strains that contained a prophage in the *sap* locus and that this prophage is absent in the majority of *C. fetus* subsp. *venerealis* bv. Intermedius strains. Almost all proteins of this prophage are hypothetical and have an unknown function, but one may speculate that the presence of this prophage influences the phenotypic difference, such as the difference in H_2S production between the *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* bv. Intermedius strains.

The differentiation between *C. fetus* subspecies goes beyond only taxonomic interest. Clinically, the subspecies have been described as different. *C. fetus* subsp. *venerealis* (including bv. Intermedius) is described as the causative agent of bovine genital cam-

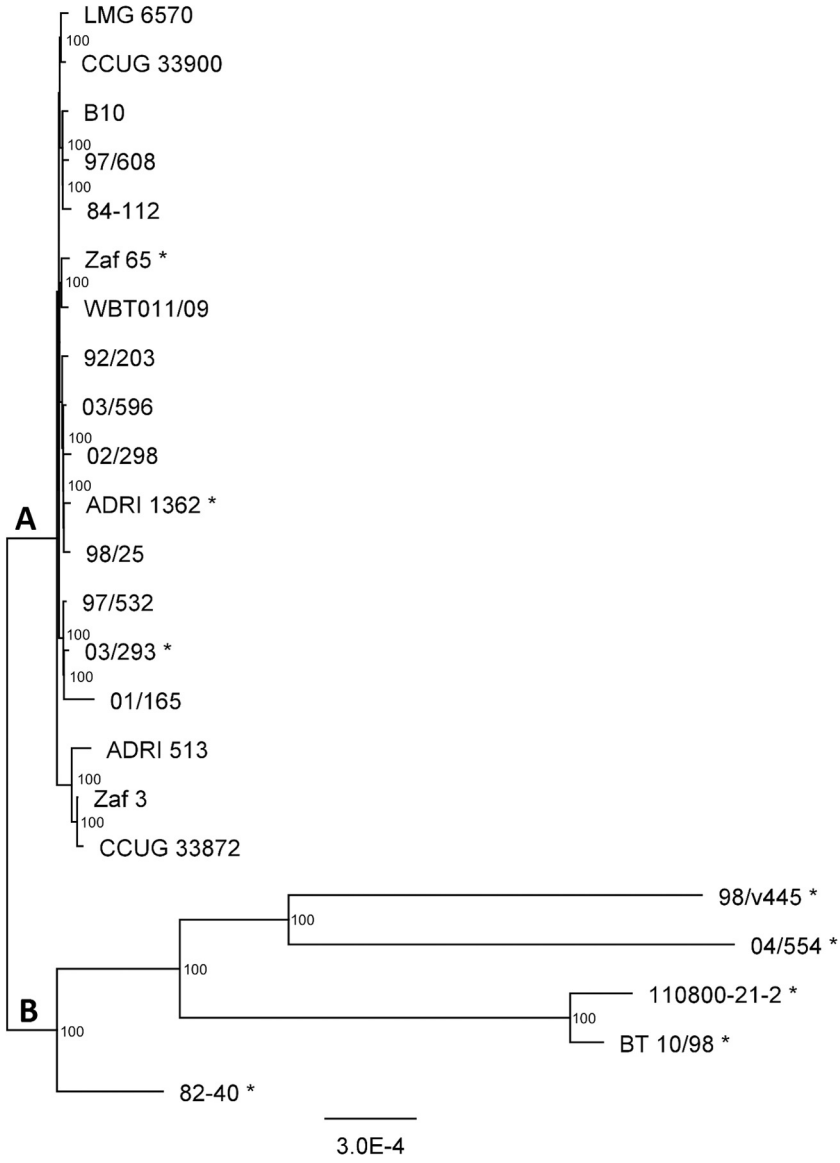

FIG 1 Phylogenetic tree of *C. fetus* strains based on the core genomes. Strains that are phenotypically identified as *C. fetus* subsp. *fetus* (Cff) are marked with an asterisk. Bootstrap supports are indicated on the branches. The scale represents the mean number of nucleotide substitutions per site.

pylobacteriosis (BGC). There is a generally accepted association between the *C. fetus* subspecies and their specific clinical features, epidemiological characteristics, and host niche specificities. Bovine products for trade must be checked for the absence of *C. fetus* subsp. *venerealis*, as stated in the *Terrestrial Animal Health Code* by the World Organization of Animal Health (OIE) (17). When *C. fetus* is detected in such a screening, subspecies identification is generally made by phenotypic assays, as described in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (18). However, there is no evidence that the phenotypic markers (glycine tolerance and H_2S production) are linked to the virulence characteristics of the *C. fetus* subspecies. Future diagnostics of *C. fetus* should preferably detect genomic characteristics associated with virulence and different host niches. The virulence genes of genomic islands present in *C. fetus* strains were described as *C. fetus* subsp. *venerealis* specific and were proposed as targets for diagnostic assays (19, 20). However, these genes are not consistently present in *C. fetus* subsp. *venerealis* genomes (13, 21) and therefore are not useful for a diagnostic assay. Pending the identification of virulence-associated genes, one should be aware that the current association between phenotype and virulence is questionable, since several phenotypically defined *C. fetus* subsp. *fetus* strains have the same genomic characteristics as *C. fetus* subsp. *venerealis* strains on the basis of core genome and accessory gene similarity, as shown in this study. The inconsistency of the phenotypes and genomic characteristics of *C. fetus* strains encourages a critical evaluation of the clinical relevance of *C. fetus* subspecies identification by phenotypic assays.

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