Homologous Recombination between Genetically Divergent *Campylobacter fetus* Lineages Supports Host-Associated Speciation

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Abstract

Homologous recombination is a major driver of bacterial speciation. Genetic divergence and host association are important factors influencing homologous recombination. Here, we study these factors for *Campylobacter fetus*, which shows a distinct intraspecific host dichotomy. *Campylobacter fetus* subspecies *fetus* (*Cff*) and *venerealis* are associated with mammals, whereas *C. fetus* subsp. *testudinum* (*Cft*) is associated with reptiles. Recombination between these genetically divergent *C. fetus* lineages is extremely rare. Previously it was impossible to show whether this barrier to recombination was determined by the differential host preferences, by the genetic divergence between both lineages or by other factors influencing recombination, such as restriction-modification, CRISPR/Cas, and transformation systems. Fortuitously, a distinct *C. fetus* lineage (ST69) was found, which was highly related to mammal-associated *C. fetus*, yet isolated from a chelonian. The whole genome sequences of two *C. fetus* ST69 isolates were compared with those of mammal- and reptile-associated *C. fetus* strains for phylogenetic and recombination analysis. In total, 5.1–5.5% of the core genome of both ST69 isolates showed signs of recombination. Of the predicted recombination regions, 80.4% were most closely related to *Cft*, 14.3% to *Cff*, and 5.6% to *C. iguaniorum*. Recombination from *C. fetus* ST69 to *Cft* was also detected, but to a lesser extent and only in chelonian-associated *Cft* strains. This study shows that despite substantial genetic divergence no absolute barrier to homologous recombination exists between two distinct *C. fetus* lineages when occurring in the same host type, which provides valuable insights in bacterial speciation and evolution.

Key words: *Campylobacter fetus*, homologous recombination, speciation, host association, reptile, whole genome sequencing.

Introduction

There are several underlying processes driving bacterial speciation. One of these processes is homologous recombination, in which genetic material is exchanged between two identical or similar molecules of DNA. Here, the effect of homologous recombination on speciation is studied in *Campylobacter fetus*, which shows several distinct genetically divergent host-associated lineages.

Campylobacter fetus is recognized as an important veterinary and occasional human pathogen (van Bergen et al. 2008; Wagenaar et al. 2014). Three *C. fetus* subspecies are currently recognized: *C. fetus* subspecies *fetus* (*Cff*) and *venerealis* (*Cfv*), which are closely related and occur in mammals, primarily ungulates, and *C. fetus* subsp. *testudinum* (*Cft*), which is genetically divergent from *Cff* and *Cfv*, and primarily occurs in reptiles (Gilbert et al. 2014). These subspecies show a strict

host dichotomy: *Cff* and *Cfv* have never been isolated from reptiles, whereas *Cft* has never been isolated from mammals, excluding occasional human cases, in which contact with reptiles is suspected (Patrick et al. 2013). The current situation shows two major dominating coherent lineages of *C. fetus* in mammals (*Cff* and *Cfv*) and reptiles (*Cft*). However, during independent studies assessing the *Campylobacter* diversity in reptiles, a genetically distinct *C. fetus* lineage, comprising multilocus sequence types 43 and 69, was obtained from captive-held red-footed tortoises (*Chelonoidis carbonaria*) in Taiwan and the Netherlands, adding up to the genetic diversity of *C. fetus* (Wang et al. 2013; Gilbert et al. 2014). Surprisingly, phylogenetic analysis based on 16S rRNA and multilocus sequence typing (MLST) showed that this lineage was highly related to mammal-associated *Cff* and *Cfv*, although it was isolated from reptiles (Wang et al. 2013).

Noteworthy, recombination was virtually absent between mammal-associated *Cff*/*Cfv* and reptile-associated *Cft*, which suggested allopatric speciation within *C. fetus* (Gilbert, Miller, Yee, Zomer, et al. 2016). A barrier to recombination was apparent, but it remained to be shown whether this was caused by the host dichotomy (mammal- or reptile-associated) or by intrinsic factors which inhibit recombination between these lineages. The *C. fetus* lineage closely related to mammal-associated *Cff* and *Cfv*, yet isolated from reptiles, might play a pivotal role in our understanding of these processes.

Based on whole genome comparison, we explore the homologous recombination between two genetically divergent bacterial lineages, which both occur naturally in a reptilian host, and provide novel insights in bacterial speciation and evolution.

Materials and Methods

Strains

Campylobacter fetus isolates 12S01208-4 and 12S01908-5 were obtained on February 23 and March 16, 2012, respectively, from a red-footed tortoise (*Chelonoidis carbonaria*) suffering from a *Mycoplasma* induced pneumonia. Isolates were grown on Columbia agar with 5% sheep blood (Oxoid, the Netherlands) in a microaerobic atmosphere (83.3% N_2, 7.1% CO_2, 3.6% H_2, and 6% O_2) at 37°C for 48 h. All other 61 *C. fetus* strains (*Cff*, 19 strains; *Cfv*, 22 strains; *Cft*, 20 strains) were identical to those used in previous studies (Gilbert, Miller, Yee, Zomer, et al. 2016; van der Graaf-van Bloois et al. 2016).

Whole Genome Sequencing

Sequencing of *C. fetus* isolates 12S01208-4 and 12S01908-5 was performed using Illumina MiSeq with 300 bp paired end reads. The reads were assembled using SPAdes 3.1.1. The average coverage was 216-354×, the number of contigs was 22-30 and the number of gaps was 23-31 for both genomes. The level of completeness (99.77%) and contamination (1.96%) was determined for both genomes based on the *Campylobacter* genus using CheckM 1.0.5. The whole genome sequences of *C. fetus* isolates 12S01208-4 and 12S01908-5 have been deposited at GenBank under accession numbers MTDX00000000 and MTDY00000000, respectively. All other *C. fetus* strains were sequenced as described previously (Gilbert, Miller, Yee, Zomer, et al. 2016; van der Graaf-van Bloois et al. 2016) and are present in GenBank.

Average Nucleotide Identity

As a measure of genomic relatedness the average nucleotide identity (ANI) was used (Konstantinidis and Tiedje 2005; Konstantinidis et al. 2006). Using the OrthoANIu tool (Lee et al. 2016), ANI values based on whole genome sequences were calculated for *C. fetus* isolates 12S01208-4 and 12S01908-5, *Cff* strains 04/554 and 82-40, *Cft* strains 03-427, 13S00388-15, 85-387, and SP3, *Cfv* strain 97/608, and *C. iguaniorum* strain 1485E.

Genome Analysis

Protein-, rRNA-, and tRNA-encoding genes were identified using Prokka (Seemann 2014). An all versus all BLAST was performed for all predicted proteins of the whole genomes (supplementary table S1, Supplementary Material online) at an E-value cutoff of $1E-6$. To determine the orthologous relationships of all proteins, protein sequences were clustered using Roary with a 75% identity cutoff (Page et al. 2015). Core genome alignment was performed using Parsnp 1.2 (Treangen et al. 2014). DNA regions present in all isolates were extracted and gaps were removed using trimAl (Capella-Gutierrez et al. 2009). Based on this core genome alignment, phylogenomic reconstruction and prediction of recombination events was performed using Gubbins (Croucher et al. 2015) with the default settings. Phylogenetic dendrograms were created using Fasttree (Price et al. 2009). A BLAST search of the predicted recombination regions of *C. fetus* ST69 isolate 12S01908-5 (supplementary information S1, Supplementary Material online) against the genomes of *Cft* strain 03-427 and *Cff* strain 82-40 and against the NCBI non redundant (nr) database was performed to search for particular recombination between these reptile-associated taxa and other species. The same procedure was performed for all predicted recombination regions of *Cft* (supplementary information S2, Supplementary Material online) and the genomes of *C. fetus* ST69 isolate 12S01908-5 and *Cff* strain 82-40. Percentage sequence identity of *C. fetus* ST69 recombination regions with *Cft* and *Cff* and percentage sequence identity of *Cft* recombination regions with *C. fetus* ST69 and *Cff* were plotted in an *x*–*y* graph using Microsoft Excel.

Multilocus Sequence Typing

The loci for the *C. fetus* multilocus sequence typing (MLST) scheme (van Bergen et al. 2005) were extracted from the whole genome of *C. fetus* isolate 12S01208-4 and submitted to the *Campylobacter* MLST database (www.pubmlst.org/campylobacter; last accessed March 17, 2016).

The 501-nt trimmed *pgm* alleles of *C. fetus* and *C. iguaniorum* strain 1485E were extracted from the *Campylobacter* MLST database and from the genome (GenBank accession number CP009043), respectively. Alignment and phylogenetic analysis, based on the neighbor-joining method with bootstrap values using 500 repetitions, was performed using MEGA 6.05.

Results

The genomes of *Campylobacter fetus* isolates 12S01208-4 and 12S01908-5 are highly similar and show a high degree of synteny. In total, 34 discriminatory SNPs were identified in the core genomes of both isolates; 88.2% (30/34) were located inside recombination regions, 11.8% (4/34) were located outside recombination regions. Both isolates belong to the same multilocus sequence type, ST69. Based on the presence of LPS-biosynthesis gene *wcbK*, encoding a putative GDP-mannose 4, 6-dehydratase, the predicted serotype of both isolates is B or AB (Kienesberger et al. 2014; Gilbert, Miller, Yee, Zomer, et al. 2016). As in other *C. fetus* lineages, the S-layer encoding *sap* genes, considered important in *C. fetus* virulence (Blaser et al. 2008), are present in *C. fetus* ST69 isolate 12S01908-5. However, no *sapCDEF* genes were detected in isolate 12S01208-4. A CRISPR/Cas system is present in *C. fetus* ST69, including two CRISPR repeat regions (30 nt spacers; 36 and 39 repeats, respectively). However, the additional locus encoding CRISPR/Cas system-associated RAMP superfamily proteins, which is well conserved in *Cff*, *Cfv*, and *Cft*, was absent from both *C. fetus* ST69 isolates (supplementary table S1, Supplementary Material online).

Of the genes specifically present in or absent from *C. fetus* ST69 only, or *C. fetus* ST69 and either *Cff/Cfv* or *Cft*, a disproportionally high number encoded proteins related to DNA uptake and defense, such as competence, transformation system, restriction-modification system, and CRISPR/Cas system proteins (supplementary table S1, Supplementary Material online).

Notably, 60 genes were exclusively shared between *C. fetus* ST69 and *Cft* strain 13S00388-15, which was isolated from *Chelonoidis denticulata*, a chelonian species closely related to *Chelonoidis carbonaria*. Most of these genes encode hypothetical proteins, but also several phage-specific proteins and likely represent a prophage.

The *tcuRABC* locus, involved in catabolism of tricarballylate (a citrate analog), which has been shown present in *Cft* and in many other reptile-associated *Campylobacter* and *Helicobacter* taxa (Gilbert, Miller, Yee, Kik, et al. 2016; Gilbert, Miller, Yee, Zomer, et al. 2016; Gilbert et al. 2017), is absent from *C. fetus* ST69.

A whole genome-based phylogeny of *C. fetus* shows that *C. fetus* ST69 is most closely related to mammal-associated *Cff* and *Cfv* (fig. 1). *Campylobacter fetus* ST69, *Cff* and *Cfv* are highly divergent from *Cft*. The average nucleotide identity (ANI) was used as a measure of genomic relatedness. The ANI between both *C. fetus* ST69 isolates was 99.96%. *Campylobacter fetus* ST69 and *Cff* showed 98% ANI, which is well above the 95% species delineation (supplementary table S2, Supplementary Material online). The ANI between *C. fetus* ST69 and *Cft* was 92%. Although this is below the species delineation, these lineages are considered conspecific based on many shared genotypic and phenotypic characteristics, including the presence of an S-layer, as examined previously for *C. fetus* (Fitzgerald et al. 2014). The ANI between *C. fetus* ST69 and reptile-associated *C. iguaniorum* was 76%.

Extensive recombination was detected in *C. fetus* ST69. In isolates 12S01208-4 and 12S01908-5, 5.1% and 5.5% of the gapless core genome was predicted to be recombined, respectively. In the *Cft* and *Cff/Cfv* genomes, on an average 2.9% and 0.4% of the core genome was predicted to be recombined, respectively. The ratio of base substitutions predicted to have been imported through recombination to those occurring through point mutation is 0.32 for *C. fetus* ST69, 0.06 for *Cft*, and 0.03 for *Cff/Cfv*, indicating that recombination is a major driver of mutation in *C. fetus* ST69.

A total of 56 different recombination regions were identified in the *C. fetus* ST69 genomes, of which two were uniquely present in isolate 12S01908-5 (fig. 1). In *Cft* and in *Cff/Cfv*, on an average 31.9 (± 5.4) and 1.5 (± 1.1) recombination regions were identified, respectively. A BLAST search against the NCBI nonredundant database showed that 80.4% (45/56) of the recombination regions in *C. fetus* ST69 were most closely related to *Cft*, 14.3% (8/56) to *Cff*, and 5.6% (3/56) to *C. iguaniorum*.

A scatter plot of the BLAST identities between the *C. fetus* strain 12S01908-5 recombination regions and *Cff* 82-40 and *Cft* 03-427 confirmed that most recombination regions were more homologous with *Cft* than with *Cff* (fig. 2*A*). On an average, the recombination regions showed 95.9% (± 2.3) homology with *Cft* and 93.2% (± 1.7) homology with *Cff*. Of the recombination regions, 98.2% (55/56) showed >87% homology with *Cff* and >89% homology with *Cft*. The homology of the recombination regions with *Cft* gradually increased from 89.4% to 99.5%, while this range was 87.3–98.7% for *Cff*. Recombination regions became scarcer with decreasing BLAST identity. Of the recombination regions, 12.5% (7/56) and 1.8% (1/56) was >98% homologous with *Cft* and *Cff*, respectively.

Two unique recombination regions were identified only in *C. fetus* ST69 isolate 12S01908-5 (indicated in blue in fig. 1). One of those regions was 99.8% (3,801/3,808 nt) homologous with *C. fetus* ST69 isolate 12S01208-4. All nucleotide

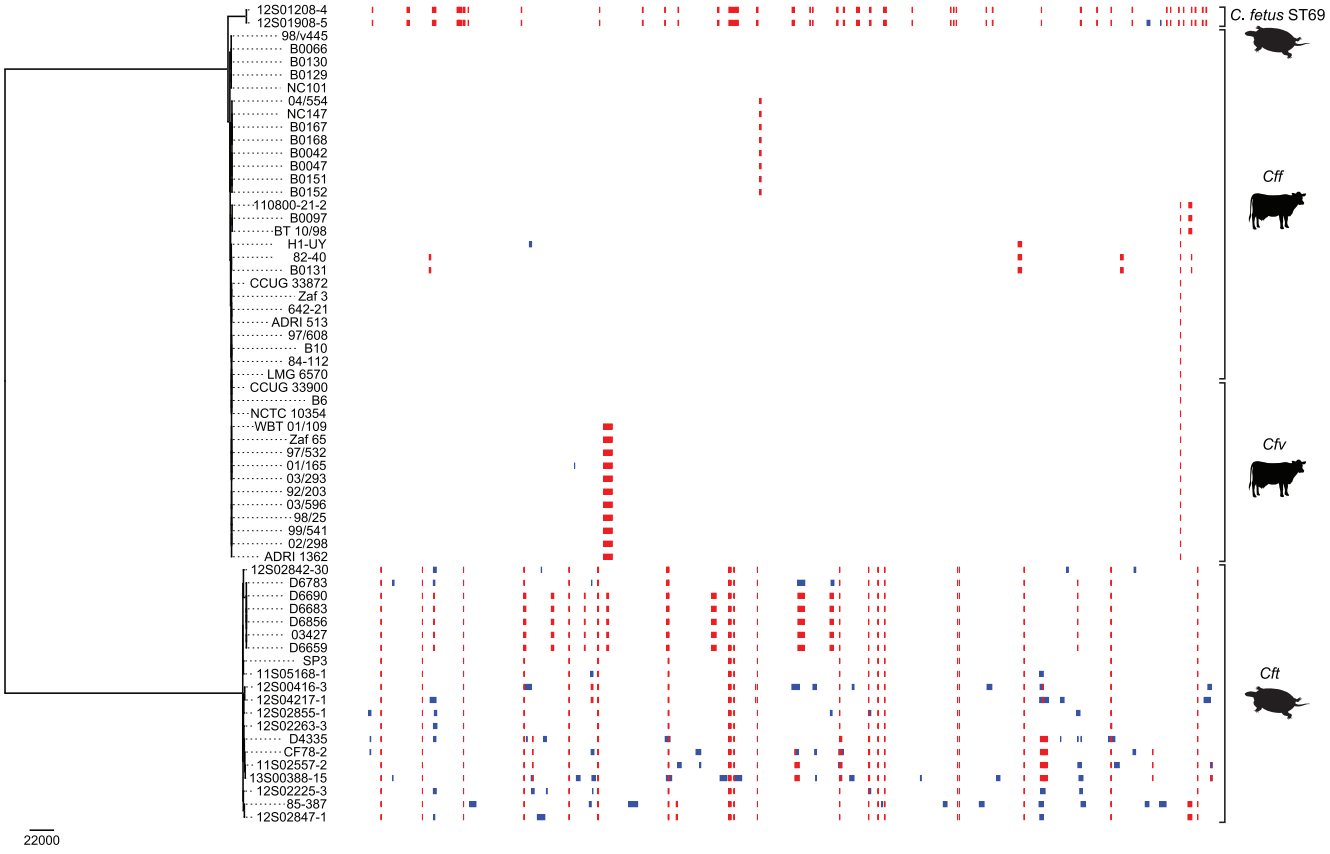

Fig. 1.—Core genome-based phylogeny for *Campylobacter fetus*. Identical recombination regions in two or more strains are indicated in red; unique recombination regions are indicated in blue. *Campylobacter fetus* ST69 is associated with reptiles (chelonians), *C. fetus* subsp. *fetus* (*Cff*) and *venerealis* (*Cfv*) are associated with mammals (primarily ungulates), and *C. fetus* subsp. *testudinum* (*Cft*) is primarily associated with reptiles.

substitutions were synonymous and did not alter the amino acid sequence of the proteins affected. The other recombined sequence was 100% (114/114 nt) homologous with reptile-associated *C. iguaniorum* strains 1485E and 2463 D. Due to this recombination, the start site of the gene encoding threonyl-tRNA synthetase and the amino acid composition of the first part of the translated protein were altered, resulting in a shorter open reading frame.

In *Cft*, recombination regions which likely originated from *C. fetus* ST69 were observed as well, but to a much lesser extent than from *Cft* to *C. fetus* ST69. Of the recombination regions extracted from *Cft*, 83.1% (108/130) showed >87% homology with *Cff* and *C. fetus* ST69. Two well-separated clusters of recombination regions which showed 52.3–75.4% and 87.5–99.9% homology with *Cff* and *C. fetus* ST69 were identified (fig. 2*B*). In total, only 2.3% (3/130) of the recombination regions likely originated from *C. fetus* ST69 (>98% homology). These regions were detected in *Cft* strains 13S00388-15 and 85-387, isolated from the chelonian species *Chelonoidis denticulata* and *Terrapene carolina*, respectively. Notably, one recombination region in *Cft* strain 85-387, showing high homology with *C. fetus* ST69, contained phosphoglucosamine mutase encoding *pgm* (*glmM*), an essential housekeeping gene which is part of the *C. fetus* MLST scheme. A dendrogram based on all *C. fetus pgm* alleles present in the pubMLST database (www.pubmlst.org/campylobacter; last accessed March 17, 2016) confirmed that *pgm* from *Cft* strain 85-387 was identical to *pgm* from *C. fetus* ST69, indicating recent recombination from the *C. fetus* ST69 lineage to *Cft* strain 85-387 (supplementary fig. S1, Supplementary Material online).

Discussion

This study shows that homologous recombination between divergent bacterial lineages occurs despite substantial genetic distance when present in the same host. As shown previously, *Cff*/*Cfv* and *Cft* show no or very few recombination between each other (Gilbert, Miller, Yee, Zomer, et al. 2016). In contrast, no absolute barrier to recombination exists between divergent *C. fetus* lineages when occurring in the same host type, as shown in this study. Apparently, a species-level divergence of 8% between both lineages is no barrier to homologous recombination. Notably, most of the recombination regions detected in both *C. fetus* ST69 and *Cft* showed highest homology with *C. fetus*, implicating that recombination is

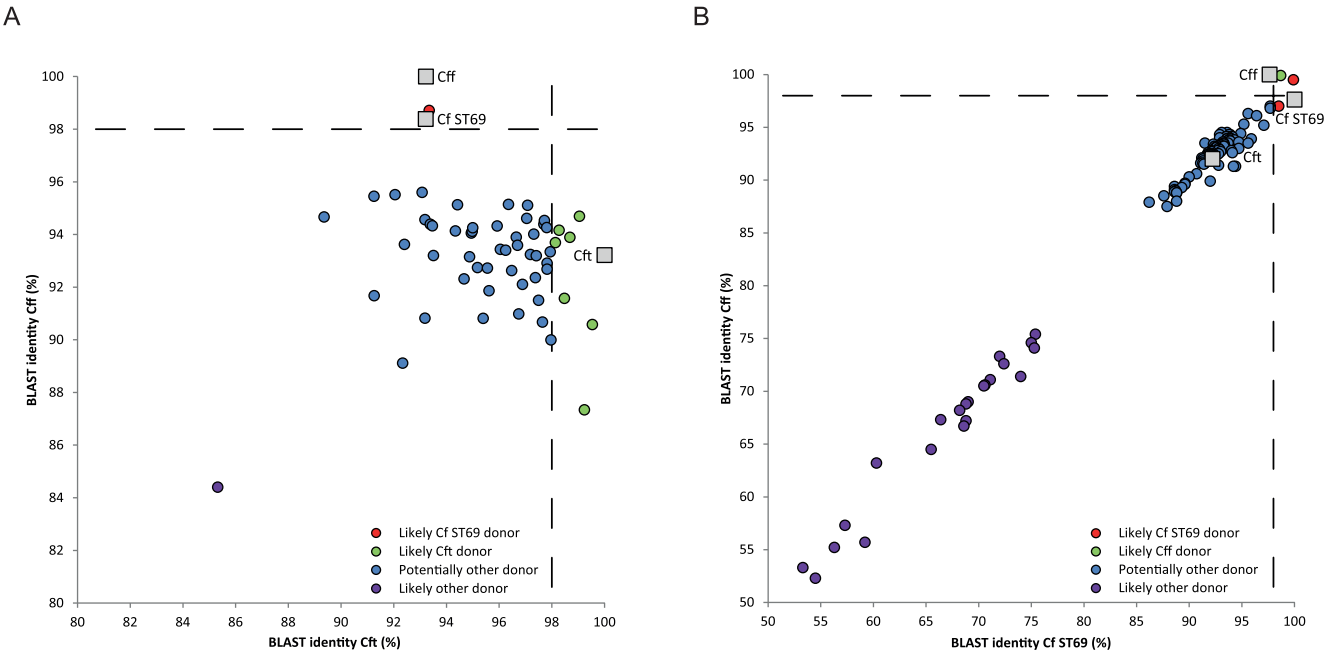

Fig. 2.—Scatter plot based on the BLAST-based nucleotide identities of all recombination regions identified in *Campylobacter fetus* ST69 (*Cf* ST69) isolate 12S01908-5 (*A*) and all *C. fetus* subsp. *testudinum* (*Cft*) strains (*B*). A BLAST search was performed for each recombination region and the genomes of *Cft* strain 03-427 and *C. fetus* subsp. *fetus* (*Cff*) strain 82-40 (*A*) and *Cff* strain 82-40 and *Cf* ST69 isolate 12S01908-5 (*B*). The nucleotide identities of the recombination regions are plotted as circles; the nucleotide identities of the reference genomes are plotted as squares. The dashed line shows the ≥98% nucleotide identity. *x*- and *y*-axis range 80–100% (*A*) or 50–100% (*B*).

occurring most frequently within *C. fetus* or a closely related *Campylobacter* lineage. Of the *C. fetus* ST69 core genome, 5.1–5.5% showed signs of recombination and the majority of the recombination regions in *C. fetus* ST69 were most closely related to *Cft*, indicating that this lineage is the most important DNA donor.

The majority of the recombination regions in *C. fetus* ST69 were most closely related to *Cft*, yet showed relative low nucleotide identity (<98%). These more divergent recombination regions likely represent ancient recombination events, followed by divergent evolution of those recombination regions based on point mutations in both donor and recipient. The gradual increase from 89.4% to 99.5% observed in the BLAST sequence identities of the *C. fetus* ST69 recombination regions with *Cft* suggests that this has been a long-term and continuous process which may still be ongoing. Indeed, parts of similar bacterial genomes can maintain the ability to recombine over long timespans before genetic isolation between two lineages is complete (Retchless and Lawrence 2007). With sets of niche-specific genes being maintained in populations that freely recombine at other loci, different parts of the genome may be genetically isolated at different times, suggesting temporal fragmentation of speciation.

Despite the high genetic similarity between both *C. fetus* ST69 isolates, small-scale differences were observed in recombination regions and gene content. As the number of discriminatory SNPs outside the recombination regions was low in the core genomes of both isolates ($n = 4$), these can be considered recent events. Furthermore, as 88.2% of the discriminatory SNPs in the core genomes of both isolates could be attributed to recombination, this can be considered the main driver of the short-scale divergence in *C. fetus* ST69. The differences in gene content could predominantly be attributed to the *sap* genes. The absence of *sapCDEF* genes in *C. fetus* ST69 isolate 12S01208-4 has been observed in other *C. fetus* strains as well and may represent a spontaneous mutation occurring in vitro (Dworkin et al. 1995; Gilbert, Miller, Yee, Zomer, et al. 2016).

Recombination also occurred in essential housekeeping genes, such as *pgm* in *Cft* strain 85-387. As *pgm* is part of the *C. fetus* MLST scheme, recombination can distort the inferences made based on MLST (Dingle et al. 2010). In addition, the observation that *Cft* strain 85-387, isolated in 1984, has an identical *pgm* allele as *C. fetus* ST69 isolates 12S01208-4 and 12S01908-5, isolated in 2012, indicates that both lineages occur together for at least 28 years and that no mutation has occurred in this allele during that period.

Reciprocal recombination between *C. fetus* ST69 and *Cft* strains from chelonian hosts suggests that recombination between both lineages primarily occurs in chelonian hosts. In addition, the presence of an identical prophage in *C. fetus*

ST69 and *Cft* strain 13S00388-15, which was isolated from *Chelonoidis denticulata*, further affirms that both *C. fetus* lineages occur in the same niche.

Interestingly, only little recombination was observed between *C. fetus* ST69 and reptile-associated *C. iguaniorum*, which showed a prevalence of 33.3% (5/15) in *Chelonoidis carbonaria* and *Chelonoidis denticulata* and was isolated from the same animal and the same samples as both *C. fetus* ST69 isolates (Gilbert et al. 2014). Recombination between *C. fetus* ST69 and *C. iguaniorum* does occur, but the genetic divergence between both species may lead to an altered protein (function), and likely reduced fitness in most cases, making it less likely that these recombination regions will be fixed in the genome over time.

Cft and *C. fetus* ST69 diverged in isolation of each other, either in space or due to intrinsic barriers to recombination. Upon contact in a shared reptilian host, recombination between both lineages occurred. Recombination between *C. fetus* ST69 and *Cft* appears to be bidirectional. However, the number and size of recombination regions was larger from *Cft* to *C. fetus* ST69 than vice versa. This asymmetry could be the result of numerical dominance of *Cft* over *C. fetus* ST69 in the reptilian intestine, which is supported by the prevalence rates (Gilbert et al. 2014), and which has been suggested previously for *C. coli* showing introgression of *C. jejuni* DNA (Sheppard et al. 2008).

Intrinsic factors explaining the high recombination frequency in *C. fetus* ST69 cannot be excluded, as genes encoding transformation and restriction-modification system proteins were disproportionally highly distributed among the genes specifically shared with either *Cft* or *Cff*/*Cfv*, or which were specifically present in *C. fetus* ST69 only. In addition, the specific absence of the additional CRISPR/Cas system-associated RAMP superfamily proteins, which have been shown well conserved in *Cff*, *Cfv*, and *Cft* (Gilbert, Miller, Yee, Zomer, et al. 2016), from *C. fetus* ST69 might favor the import of exogenous DNA and enable a higher recombination frequency.

Homologous recombination acts as a force of coherence between both lineages and can counteract speciation (Fraser et al. 2007). When occurring in identical host types recombination between divergent *C. fetus* lineages occurs. However, when occurring in different host types, that is, mammalian or reptilian, recombination is virtually absent (Gilbert, Miller, Yee, Zomer, et al. 2016). This study shows that no obvious barriers to homologous recombination exist between two genetically divergent bacterial lineages when colonizing the same host type. In this case, recombination is rather predicted by host specificity than by sequence divergence, supporting allopatric speciation based on host type.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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