Supplementary Information for

Profiling *Fusobacterium* infection at high taxonomic resolution reveals lineage-specific correlations in colorectal cancer

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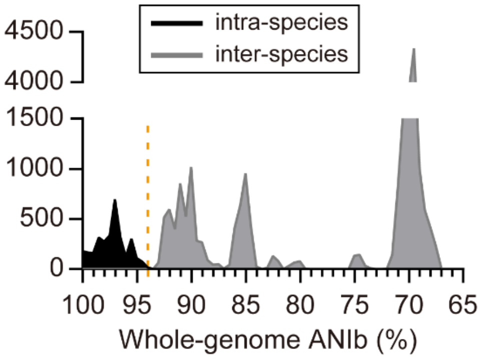


Figure S1. Whole-genome ANIb analysis effectively defined species. Distribution of pairwise intra-species (*n* =3,068) and inter-species (*n* =20,802) ANIbs of 157 *Fusobacterium* genomes is shown. Orange highlights species boundaries (94% ANIb). The analysis considered the four *F. nucleatum* subspecies as separate species. ANIb, average nucleotide identity calculated with BLAST.

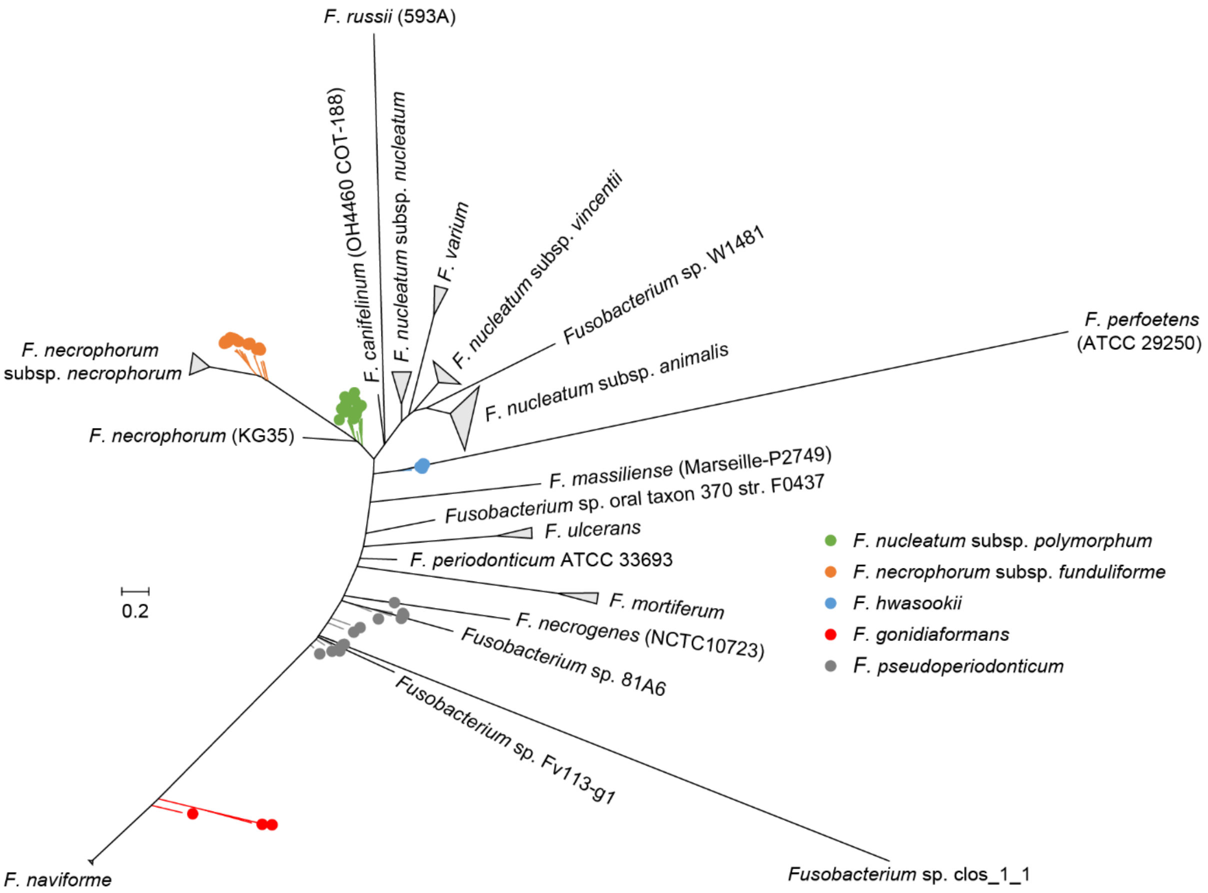

F. russii (593A)
F. canifelinum (OH4460 COT-188)
F. nucleatum subsp. nucleatum
F. varium
F. nucleatum subsp. vincentii
Fusobacterium sp. W1481
F. perfoetens (ATCC 29250)
F. nucleatum subsp. animalis
F. necrophorum subsp. necrophorum
F. necrophorum (KG35)
F. massiliense (Marseille-P2749)
Fusobacterium sp. oral taxon 370 str. F0437
F. ulcerans
F. periodonticum ATCC 33693
F. mortiferum
F. necrogenes (NCTC10723)
Fusobacterium sp. 81A6
Fusobacterium sp. Fv113-g1
Fusobacterium sp. clos_1_1
F. naviforme
0.2
● F. nucleatum subsp. polymorphum
● F. necrophorum subsp. funduliforme
● F. hwasookii
● F. gonidiaformans
● F. pseudoperiodonticum

Figure S2. Whole-genome phylogeny produced a *Fusobacterium* taxonomy similar to that produced by ANIb. The 157 *Fusobacterium* genomes were used. The tree was generated by using kSNP3 with the maximum likelihood algorithm. Strain names are given in parentheses for the species with only one sequenced genome available. Branches of the same species/subspecies are compressed as applicable or denoted with dots of the same colour. The colour scheme is shown in the figure.

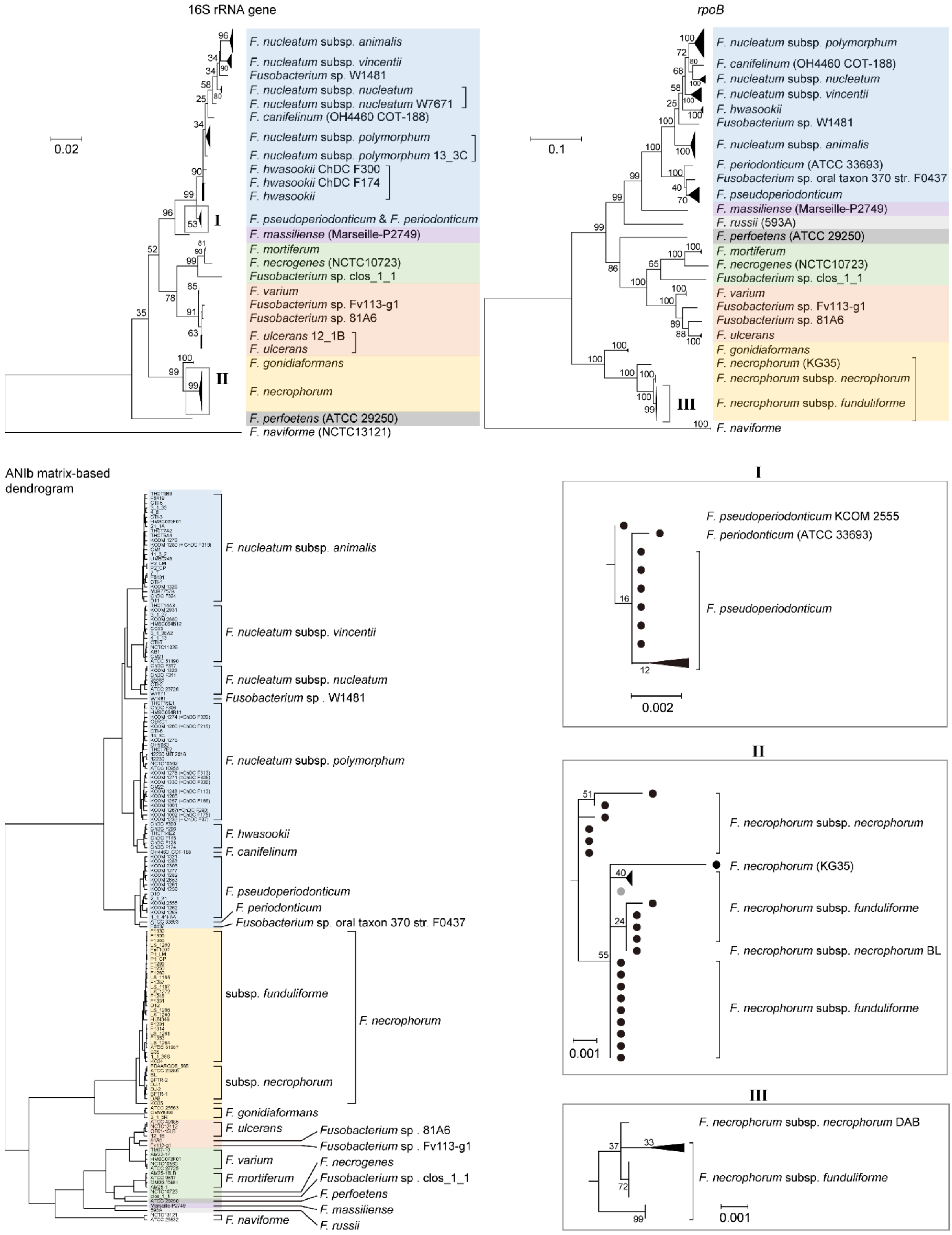

16S rRNA gene
96
34
90
34
58
80
25
34
0.02
90
99
96
53
I
52
81
93
99
85
78
91
35
63
100
99
II
99
F. nucleatum subsp. animalis
F. nucleatum subsp. vincentii
Fusobacterium sp. W1481
F. nucleatum subsp. nucleatum
F. nucleatum subsp. nucleatum W7671
F. canifelinum (OH4460 COT-188)
F. nucleatum subsp. polymorphum
F. nucleatum subsp. polymorphum 13_3C
F. hwasookii ChDC F300
F. hwasookii ChDC F174
F. hwasookii
F. pseudoperiodonticum & F. periodonticum
F. massiliense (Marseille-P2749)
F. mortiferum
F. necrogenes (NCTC10723)
Fusobacterium sp. clos_1_1
F. varium
Fusobacterium sp. Fv113-g1
Fusobacterium sp. 81A6
F. ulcerans 12_1B
F. ulcerans
F. gonidiaformans
F. necrophorum
F. perfoetens (ATCC 29250)
F. naviforme (NCTC13121)
rpoB
100
72
80
68
100
58
100
25
100
100
0.1
100
100
100
99
100
40
99
70
99
86
65
100
100
100
100
100
89
88
100
100
100
100
100
100
99
III
100
F. nucleatum subsp. polymorphum
F. canifelinum (OH4460 COT-188)
F. nucleatum subsp. nucleatum
F. nucleatum subsp. vincentii
F. hwasookii
Fusobacterium sp. W1481
F. nucleatum subsp. animalis
F. periodonticum (ATCC 33693)
Fusobacterium sp. oral taxon 370 str. F0437
F. pseudoperiodonticum
F. massiliense (Marseille-P2749)
F. russii (593A)
F. perfoetens (ATCC 29250)
F. mortiferum
F. necrogenes (NCTC10723)
Fusobacterium sp. clos_1_1
F. varium
Fusobacterium sp. Fv113-g1
Fusobacterium sp. 81A6
F. ulcerans
F. gonidiaformans
F. necrophorum (KG35)
F. necrophorum subsp. necrophorum
F. necrophorum subsp. funduliforme
F. naviforme
ANIb matrix-based dendrogram
F. nucleatum subsp. animalis
F. nucleatum subsp. vincentii
F. nucleatum subsp. nucleatum
Fusobacterium sp . W1481
F. nucleatum subsp. polymorphum
F. hwasookii
F. canifelinum
F. pseudoperiodonticum
F. periodonticum
Fusobacterium sp. oral taxon 370 str. F0437
subsp. funduliforme
F. necrophorum
subsp. necrophorum
F. gonidiaformans
F. ulcerans
Fusobacterium sp . 81A6
Fusobacterium sp . Fv113-g1
F. varium
F. necrogenes
F. mortiferum
Fusobacterium sp . clos_1_1
F. perfoetens
F. massiliense
F. naviforme
F. russii
I
F. pseudoperiodonticum KCOM 2555
F. periodonticum (ATCC 33693)
16
F. pseudoperiodonticum
12
0.002
II
51
F. necrophorum subsp. necrophorum
40
F. necrophorum (KG35)
F. necrophorum subsp. funduliforme
24
55
F. necrophorum subsp. necrophorum BL
F. necrophorum subsp. funduliforme
0.001
III
F. necrophorum subsp. necrophorum DAB
37
33
F. necrophorum subsp. funduliforme
72
99
0.001

Figure S3. Phylogenetic trees of the *Fusobacterium* 16S rRNA gene and *rpoB* were largely similar and the *rpoB*-based tree produced better delineation. Complete gene sequences available in the 157 sequenced genomes were used for analysis (144 16S rRNA gene and 157

rpoB sequences). Strain names are given in parentheses for the species with only one sequence available. The I-III grey boxes correspond to the I-III labels in the trees. Strain names are also provided for those that could not be compressed together. Branches of the same species/subspecies or otherwise illustrated in the boxes are compressed as applicable. Stains on indistinctive edges are denoted by dots. The ANIb matrix-based dendrogram from Fig. 1 was also included for comparison. Species that could consistently form a lineage across the 16S rRNA gene tree, *rpoB* tree and ANIb-based dendrogram are shaded with the same colour. The colour scheme is identical to that used in Figs. 3, S5 and S7. ANIb, average nucleotide identity calculated with BLAST.

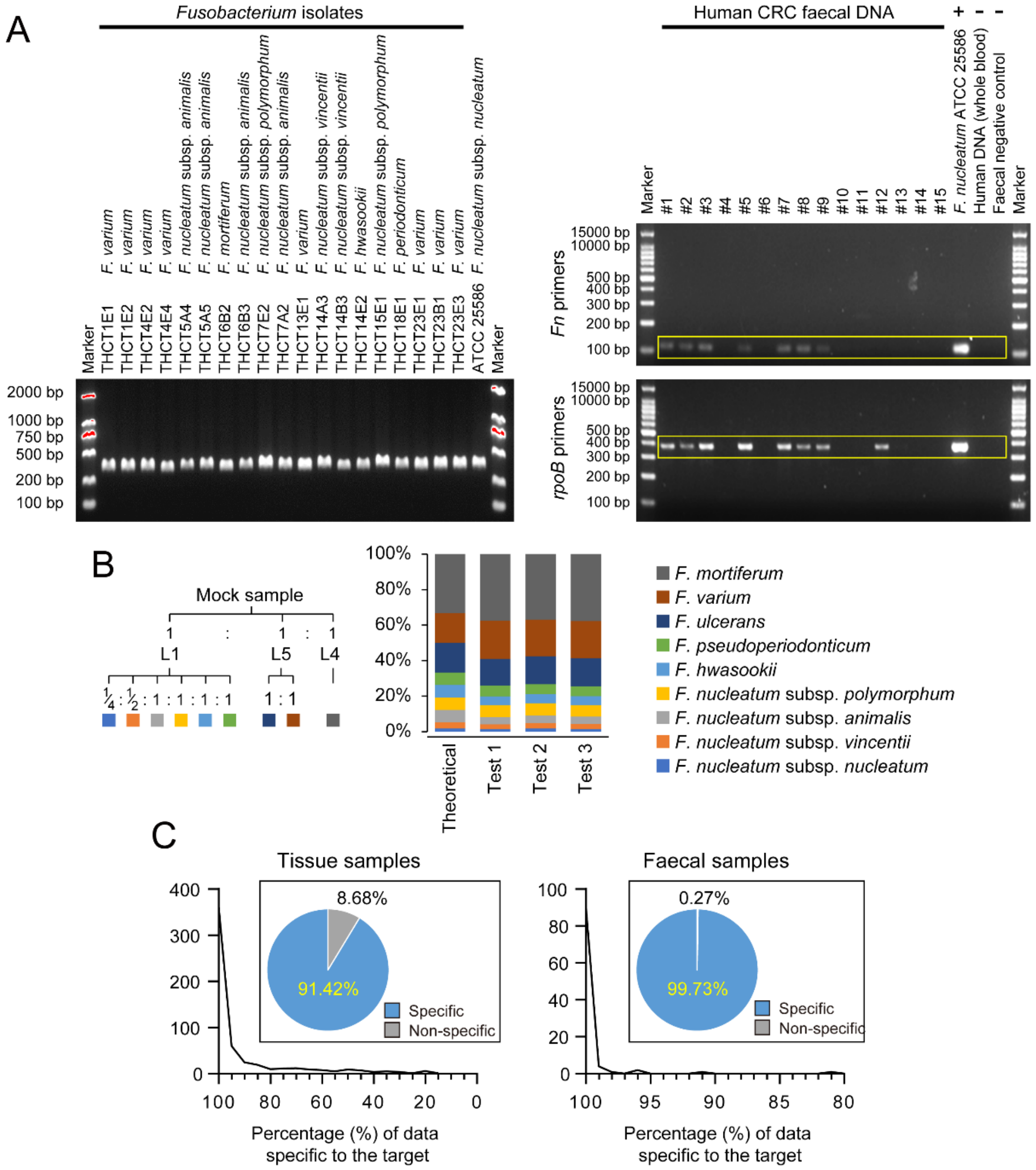


Figure S4. Assessment of amplification performance. (A) The designed primers amplified a specific band in *Fusobacterium* isolates and faecal samples from colorectal cancer (CRC) patients. For faecal samples, amplification with the *F. nucleatum* detection primers (*Fn* primers) was performed in parallel for reference. The *Fn* primers were the universal primers used for *F. nucleatum* detection (Fn-F: 5'-CAACCATTACTTTAACTCTACCATGTTCA-3' and Fn-R: 5'-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-3') (Castellarin, *et al*, *Genome Res* 2012; Mima, *et al*, *JAMA Oncol* 2015), while the *rpoB* primers were the universal primers designed for the selected *rpoB* region. *F. nucleatum* subsp. *nucleatum* ATCC 25586 DNA was

used as a positive control (“+”). Human whole-blood DNA negative control and a faecal negative control were also used (“-”). Experiments were conducted in triplicate and representative gel images are shown. (B) Design of a mock sample and its composition detected by FrpoB-seq (in triplicate). (C) The FrpoB-seq data indicated high amplification specificity in the test samples. Distribution of the percentages of sequencing data specific to the *Fusobacterium rpoB* target in tissue and faecal samples are shown, with overall percentages given in the boxes. Colour schemes are denoted in the figure.

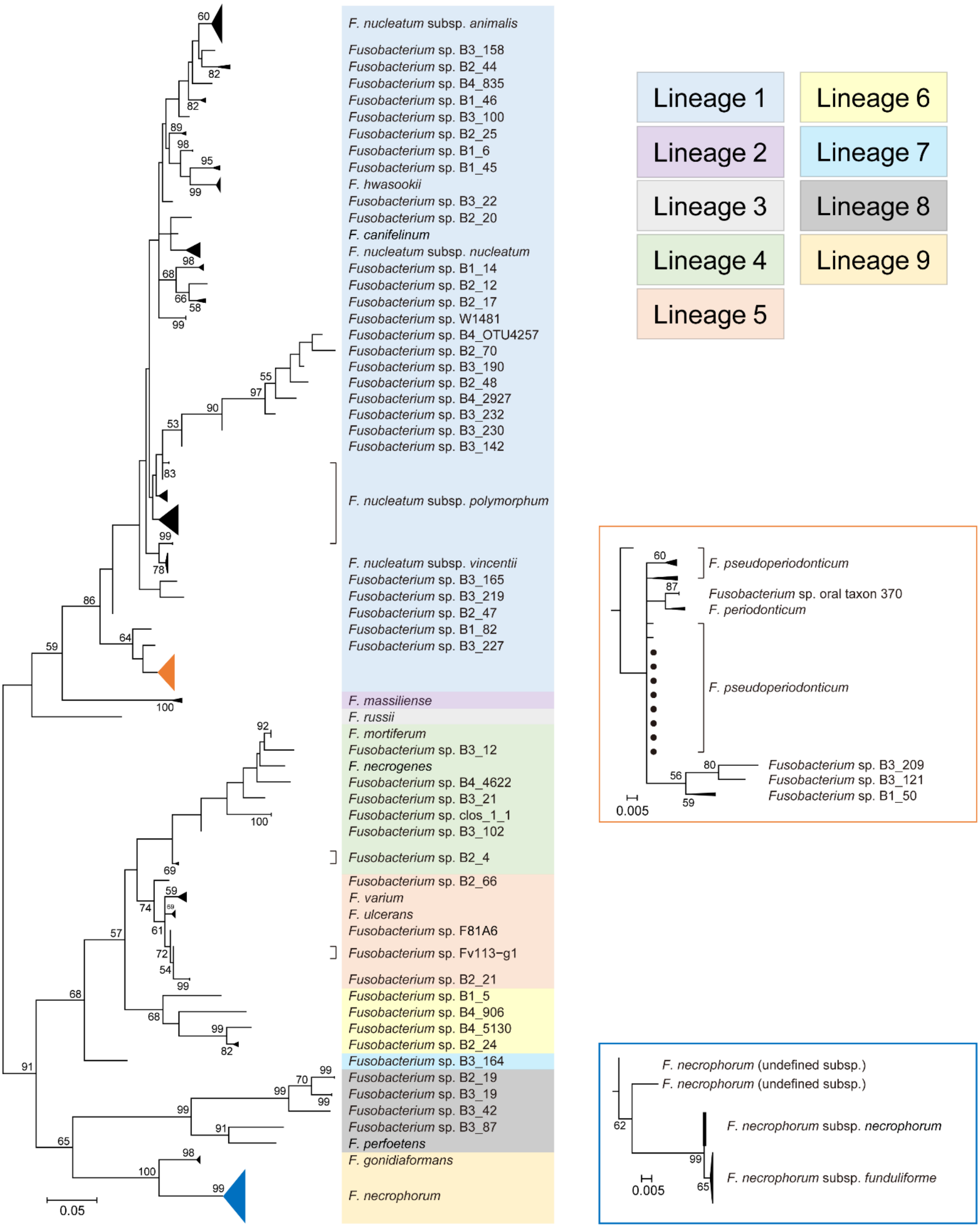
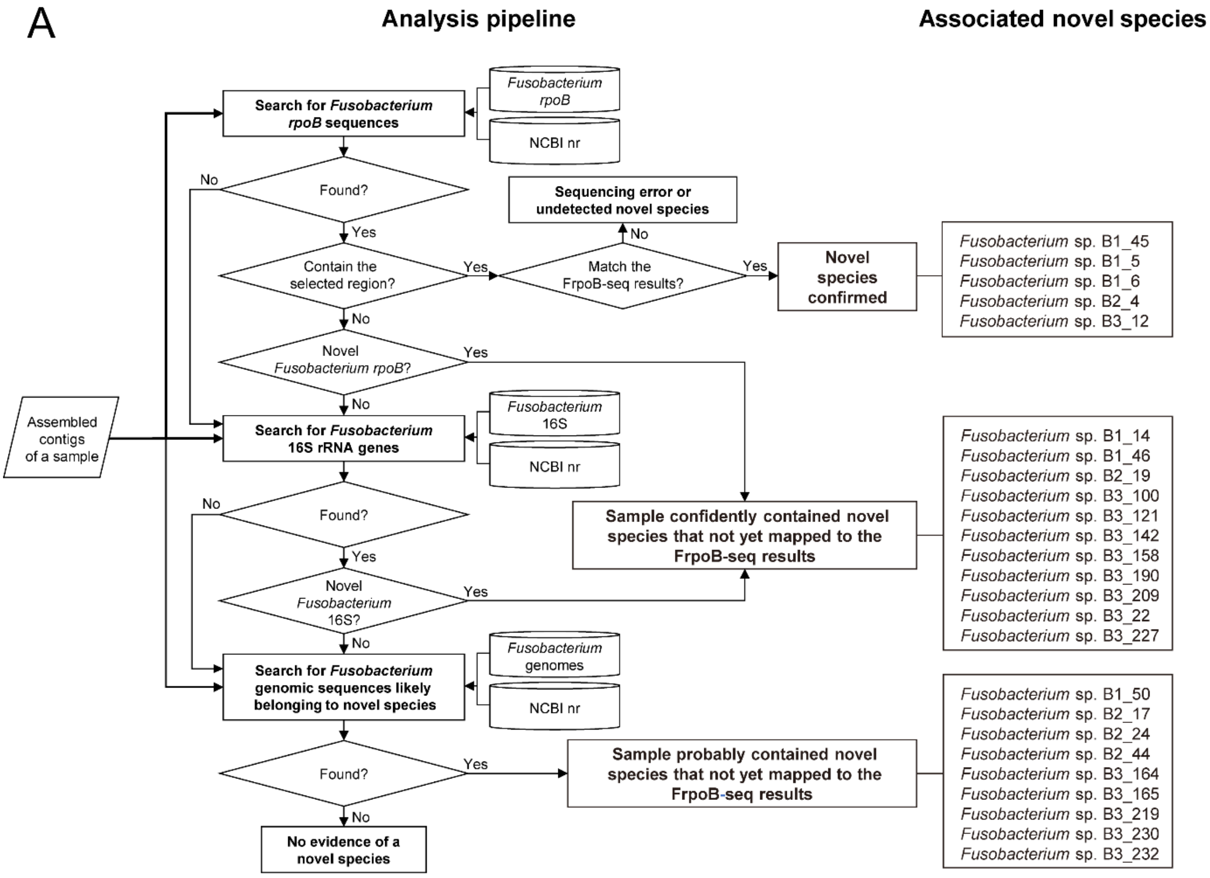


Figure S5. The *rpoB*-based approach identified a striking number of new species and the phylogenetic tree of all *Fusobacterium* species based on the selected *rpoB* region delineated them into 9 lineages. Both newly identified and previously known species were included in this analysis. The tree was based on the corresponding sequences in the genomes

or those obtained via FrpoB-seq. Branches of the same species/subspecies or those otherwise illustrated in the boxes are compressed as applicable. The boxes correspond to the triangles in the tree by same colours (orange and blue). Stains on indistinctive edges are denoted by dots. Lineages are denoted by different colours with the legend shown in the figure.


A
Analysis pipeline
Associated novel species
Assembled contigs of a sample
Search for *Fusobacterium* *rpoB* sequences
Fusobacterium *rpoB*
NCBI nr
Found?
No
Yes
Contain the selected region?
Yes
Match the FrpoB-seq results?
No
Sequencing error or undetected novel species
Yes
Novel species confirmed
Fusobacterium sp. B1_45
Fusobacterium sp. B1_5
Fusobacterium sp. B1_6
Fusobacterium sp. B2_4
Fusobacterium sp. B3_12
No
Novel *Fusobacterium rpoB*?
Yes
No
Search for *Fusobacterium* 16S rRNA genes
Fusobacterium 16S
NCBI nr
Found?
No
Yes
Novel *Fusobacterium* 16S?
Yes
No
Sample confidently contained novel species that not yet mapped to the FrpoB-seq results
Fusobacterium sp. B1_14
Fusobacterium sp. B1_46
Fusobacterium sp. B2_19
Fusobacterium sp. B3_100
Fusobacterium sp. B3_121
Fusobacterium sp. B3_142
Fusobacterium sp. B3_158
Fusobacterium sp. B3_190
Fusobacterium sp. B3_209
Fusobacterium sp. B3_22
Fusobacterium sp. B3_227
Search for *Fusobacterium* genomic sequences likely belonging to novel species
Fusobacterium genomes
NCBI nr
Found?
Yes
Sample probably contained novel species that not yet mapped to the FrpoB-seq results
Fusobacterium sp. B1_50
Fusobacterium sp. B2_17
Fusobacterium sp. B2_24
Fusobacterium sp. B2_44
Fusobacterium sp. B3_164
Fusobacterium sp. B3_165
Fusobacterium sp. B3_219
Fusobacterium sp. B3_230
Fusobacterium sp. B3_232
No
No evidence of a novel species

B

Southern Chinese population cohort, n = 556 (Yeoh, *et al*, 2020)
(average ~7.5 Gbp)

Accession(s)	Detected novel species
SRR10680692	*Fusobacterium* sp. B1_6
SRR10680388, SRR10680416, SRR10680433, SRR10680435, SRR10680467, SRR10680471, SRR10680498, SRR10680545, SRR10680609, SRR10680651, SRR10680707, SRR10680730, SRR10680744, SRR10680761, SRR10680770, SRR10680788, SRR10901570	*Fusobacterium* sp. B2_4
SRR10680413	*Fusobacterium* sp. B2_19
SRR10680370, SRR10680429	*Fusobacterium* sp. B2_24
SRR10680577, SRR10680758, SRR10680776, SRR10680790, SRR10680868	*Fusobacterium* sp. B3_19
SRR10680530, SRR10680741	*Fusobacterium* sp. B3_21
SRR10680858	*Fusobacterium* sp. B3_42
SRR10680663	*Fusobacterium* sp. B3_102
SRR10680480	*Fusobacterium* sp. B2_4, *Fusobacterium* sp. B2_19
SRR10680611	*Fusobacterium* sp. B2_19, *Fusobacterium* sp. B3_19
SRR10680861	*Fusobacterium* sp. B2_4, *Fusobacterium* sp. B3_19
SRR10680767	*Fusobacterium* sp. B3_19, *Fusobacterium* sp. B3_102
SRR10680332, SRR10680691, SRR10680746	*Fusobacterium* sp. B2_4 / *Fusobacterium* sp. B3_102
SRR13061017	*Fusobacterium* sp. B1_6 / *Fusobacterium* sp. B2_48
SRR10680361	putative novel species (~94% identity to *Fusobacterium* sp. B2_19)
SRR10680330	putative novel species (~96% identity to *Fusobacterium*
SRR10680491	putative novel species (~96% identity to *Fusobacterium* sp. B3_42)

Ultra-deep whole-metagenomic shotgun sequencing (Korea) cohort, n = 106 (Kim, *et al*, 2021)
(average >30 Gbp)

Accession	Detected novel species
SRR13061017	*Fusobacterium* sp. B2_48 / *Fusobacterium* sp. B1_6

Figure S6. Identification of putative novel *Fusobacterium* species with metagenomic sequencing data. (A) Identification in a subset (n =35, see Dataset S3) of the collected faecal

samples covering 25 putative novel species. There were 26 putative novel species identified in faecal samples, but one (*Fusobacterium* sp. B1_82) could not be assessed due to that the sample containing it had insufficient DNA for sequencing. A three-step analysis pipeline was used. The assembled contigs of a sample were aligned with the full-length *Fusobacterium rpoB* and compared against the NCBI nucleotide (nr) database to search for *Fusobacterium*-specific *rpoB* sequences, which were then aligned with all available *rpoB* sequences (from the genomes and the FrpoB-seq data) to check if they could be mapped to those of the novel species. If the selected region used for FrpoB-seq was not covered in the metagenomic data, the available *rpoB* fragments were used to assess if they belonged to novel species at an identity cut-off of <96%, a species boundary found in Fig. 2A. *Fusobacterium*-specific 16S rRNA gene sequences were also search similarly and used to assess if they belonged to novel species at a cut-off of <98.5%, a stringent species boundary found in Fig. 2A. Finally, *Fusobacterium*-specific genomic sequences besides of *rpoB* and 16S rRNA gene, which had no non-*Fusobacterium* hit at a >20% coverage in the NCBI nr database, were search similarly and used to assess if they belonged to novel species. The criteria were that their identities to the hits of known species were <89% (a minus five of the ANIb boundary) with >50% coverages and also smaller than the intra-species identities of their alleles (if available). The identification results are given on the right side. Notably, scenario of sequencing error or existence of other novel species undetected by FrpoB-seq as denoted in the pipeline was not found. (B) Identification in public metagenomic datasets. *Fusobacterium*-specific *rpoB* sequences were selected and aligned against known sequences. The detected novel species that mapped to those identified by FrpoB-seq are given along with the corresponding sample accession numbers.

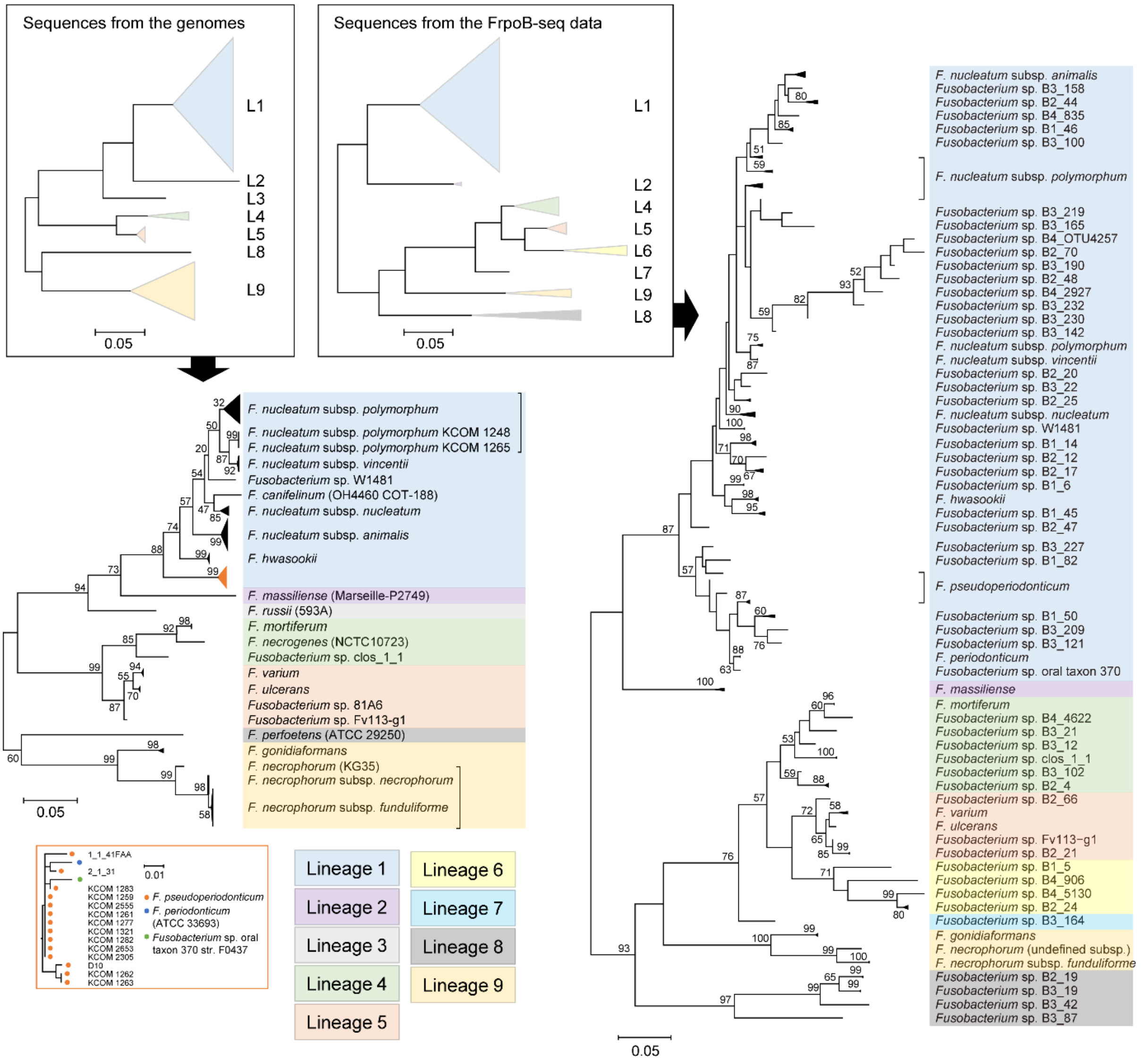


Figure S7. The lineage classification results were concordant between the phylogenetic trees based on the sequences of the selected *rpoB* region from the genomes and the FrpoB-seq data. Note that the tree of the former is identical to that shown in Fig. 2C. In that tree, branches of the same species/subspecies or those otherwise illustrated in the orange box (corresponding to the oragnge triangle). There was no available genome of L6 and L7 species, and in the FrpoB-seq data, no L3 species was detected. Lineages are denoted by different colours with the legend shown in the figure.

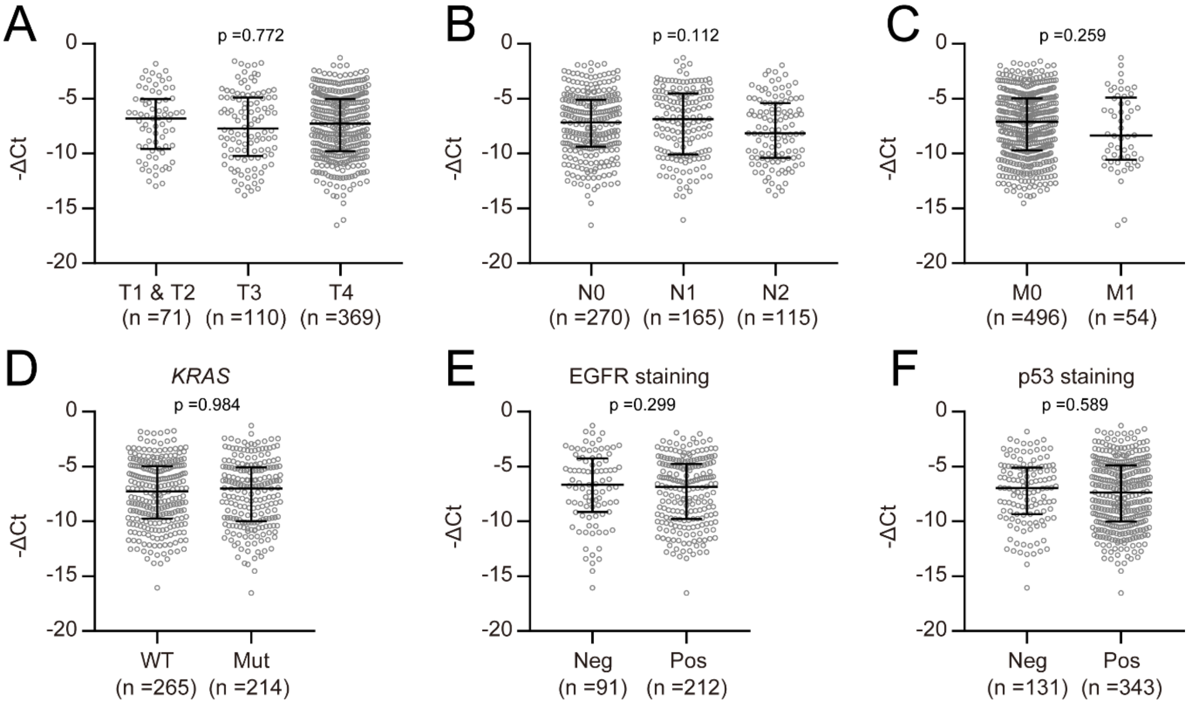


Figure S8. Relative abundance of *Fusobacterium* in tumour tissues did not vary with any of the examined pathological features of CRC. (A), (B) and (C) Comparison by T, N, and M stages, respectively. (D) Comparison by *KRAS* mutation status. WT, wild type; Mut, mutation (E) and (F) Comparison of immunohistochemical staining results for EGFR and p53, respectively. Neg, negative; Pos, positive. For (A) and (B), Kruskal-Wallis test followed by Dunn's multiple comparison test. The p values of Kruskal-Wallis test are shown. For (C)–(F), Mann-Whitney test. Individual data points are shown along with the medians and interquartile ranges. All statistical analyses are two-sided where applicable.

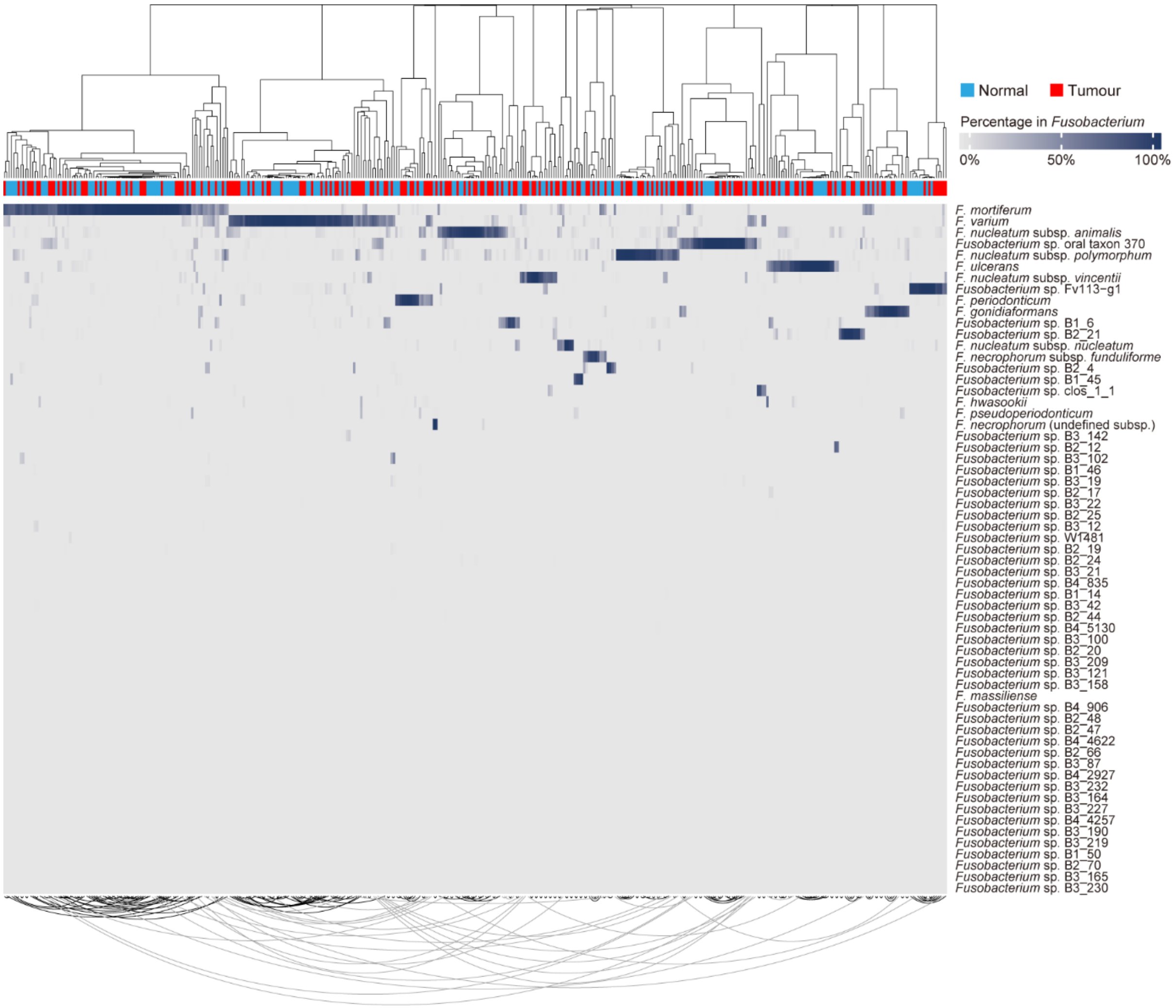


Figure S9. ***Fusobacterium* species compositions of 201 paired tumour and normal tissues.** Percentages in the *Fusobacterium* community of each sample are presented as a heatmap. The colour scheme is shown in the figure. Paired samples are connected by lines. The grey lines indicate the paired samples located on separate major branches. The black lines indicate otherwise.

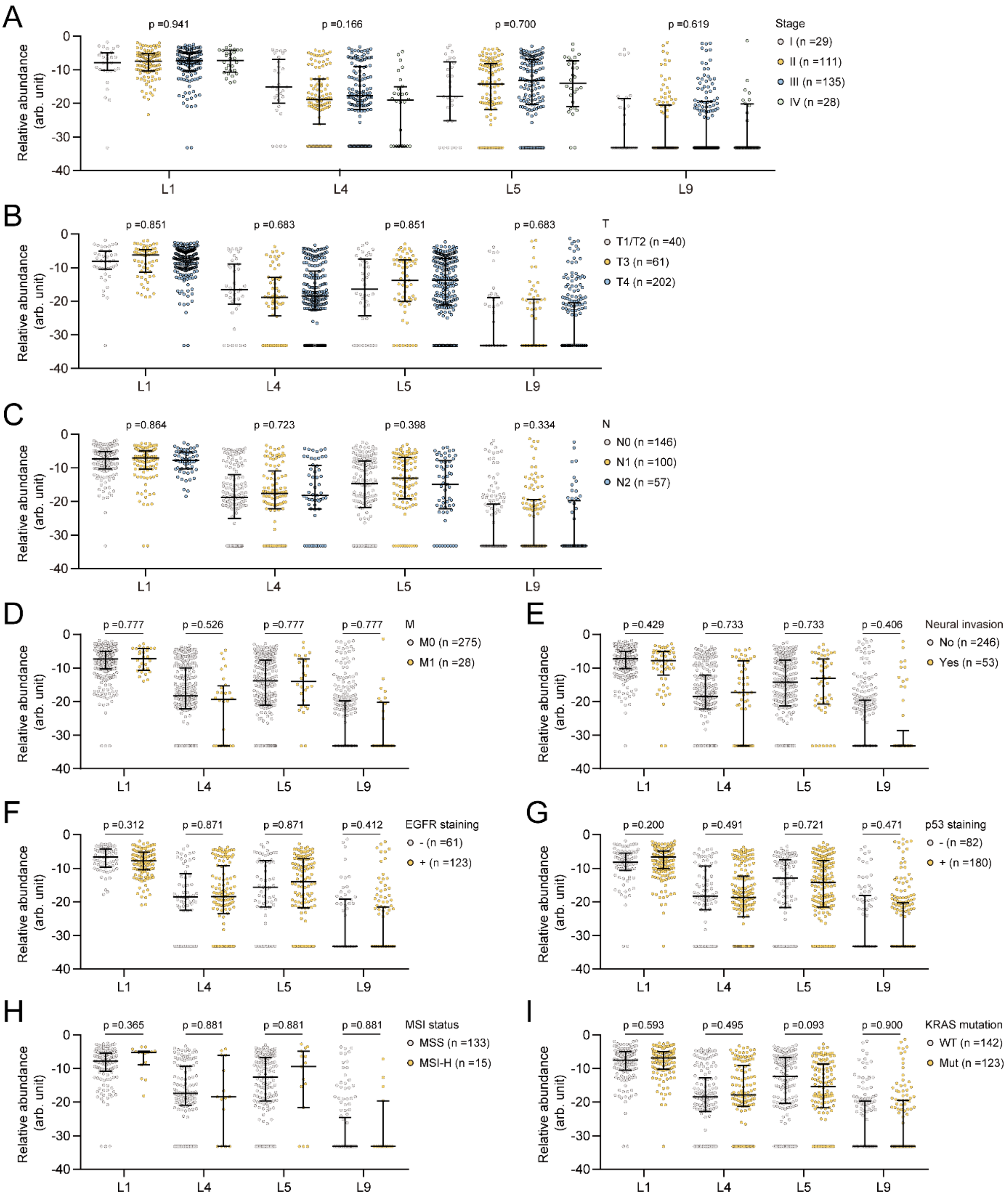


Figure S10. Associations between lineage abundance and pathological characteristics. (A) Comparison by stage. (B), (C) and (D) Comparison by tumour, node, and metastasis (TNM) stages, respectively. (E), (F), (G), (H) and (I) Comparison by neural invasion status, EGFR staining result, p53 staining result, MIS status and *KRAS* mutation status, respectively. For (A)-(C), Kruskal-Wallis test followed by Dunn's multiple comparison test was used for each lineage.

Benjamini-Hochberg correction was then applied to correct the p values of the Kruskal-Wallis tests and the adjusted p values are shown for each lineage. For (D)-(I), Mann-Whitney test was used for each lineage and Benjamini-Hochberg correction was then applied. Adjusted p values are shown. Individual data points are shown along with the medians and interquartile ranges. arb. unit, arbitrary unit. All statistical analyses are two-sided where applicable.

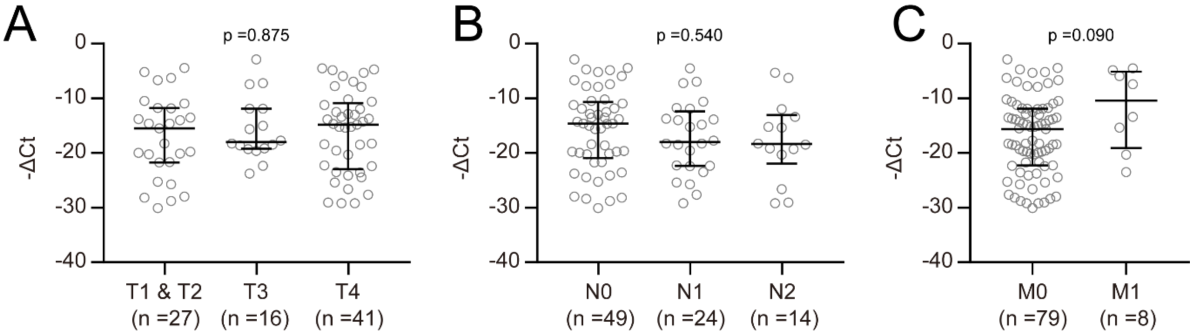


Figure S11. Relative abundance of *Fusobacterium* in faecal samples from CRC patients compared by tumour, node, metastasis (TNM) stage. (A), (B) and (C) Comparison by T, N, and M stages, respectively. For (A) and (B), Kruskal-Wallis test followed by Dunn's multiple comparison test. The p values of Kruskal-Wallis test are shown. For (C), Mann-Whitney test. Individual data points are shown along with the medians and interquartile ranges. All statistical analyses are two-sided where applicable.

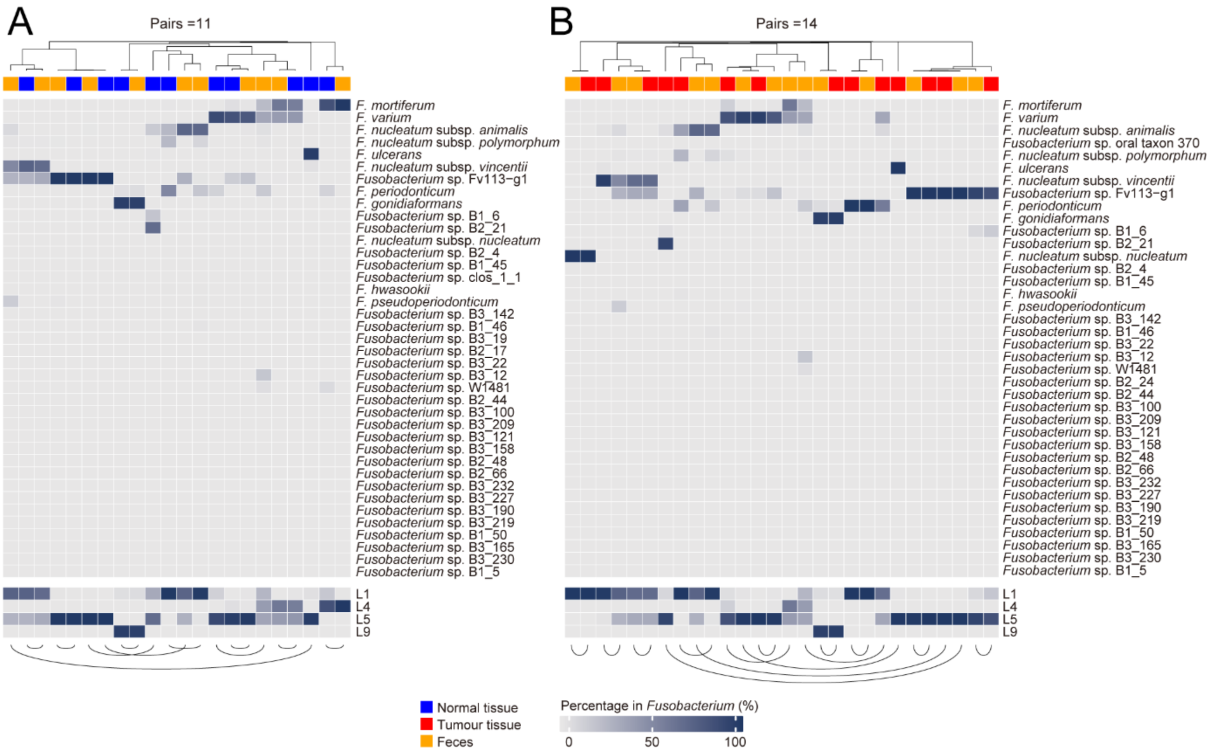


Figure S12. ***Fusobacterium* species compositions in faecal samples and matched normal or tumour tissues.** (A) Results for patients with available FrpoB-seq data for faecal samples and matched normal tissues. (B) Results for patients with available FrpoB-seq data for faecal samples and matched tumour tissues. Percentages of specific species within the *Fusobacterium* community of each sample are presented as a heatmap. Paired samples are connected by lines.

Table S1. Bacterial strains used in this study.

Species	Strain	Reference
F. nucleatum subsp. *nucleatum*	ATCC 25586	ATCC
F. nucleatum subsp. *animalis*	THCT5A4 (CCTCC M 2019366)	This study
	THCT6B3 (CCTCC M 2019367)	This study
	THCT7A2 (CCTCC M 2019365)	This study
	THCT5A5	This study
F. nucleatum subsp. *polymorphum*	THCT7E2 (CCTCC M 2019364)	This study
	THCT15E1 (CCTCC M 2019362)	This study
F. nucleatum subsp. *vincentii*	THCT14A3 (CCTCC M 2019363)	This study
	THCT14B3	
F. hwasookii	THCT14E2 (CCTCC M 2019361)	This study
F. varium	THCT1E1	This study
	THCT1E2	This study
	THCT4E2	This study
	THCT4E4	This study
	THCT13E1	This study
	THCT23E1	This study
	THCT23B1	This study
	THCT23E3	This study
F. mortiferum	THCT6B2	This study
F. pseudoperiodonticum	THCT18E1	This study
F. ulcerans	ATCC 49185	ATCC

Table S2. Putative non-specific amplification with the designed universal primers

Species of hit	Habitat/known origin of isolation
Leptotrichia buccalis (Fusobacteriales)	human oral and vaginal cavities
Leptotrichia sp. oral taxon 847 (Fusobacteriales)	human oral cavity
Leptotrichia goodfellowii (Fusobacteriales)	human faeces and oral and intestinal flora
Sebaldella termitidis (Fusobacteriales)	termite intestine
Sneathia amnii (Fusobacteriales)	pathogen of the female urogenital tract
Caviibacter abscessus/*Streptobacillus moniliformis* (Fusobacteriales)	guinea pigs
Alkaliflexus imshenetskii	soda lake
Psychrilyobacter atlanticu (Fusobacteriales)	marine environments
Sneathia sanguinegens (Fusobacteriales)	human oral cavity and urogenital tract
Streptobacillus notomytis (Fusobacteriales)	rat (unusual in human)
Leptotrichia trevisanii (Fusobacteriales)	NA
Labilibacter marinus	marine sediments
Photobacterium damselae	marine animals
Alkalitalea saponilacus	Soap Lake
Clostridium oryzae	soil
Enterococcus hirae	zoonotic pathogen (unusual in human)
Bacillus mycoides	soil
Roseovarius mucosus	diatom
Bizionia argentinensis	marine environments
Mycoplasma hyosynoviae	pig

Species belonging to the order Fusobacteriales are denoted in parentheses. Information of habitat/known origin of isolation was retrieve from the NCBI database. NA, not available.

Table S3. Annotation of non-specific sequences obtained by FrpoB-seq

Species in which the non-specific sequences were found or had the best hits
Homo sapiens
Leptotrichia buccalis
Leptotrichia hongkongensis
Leptotrichia trevisanii
Leptotrichia wadei
Leptotrichia sp. oral taxon 498
Leptotrichia sp. oral taxon 212
Clostridium
Eubacterium
Akkermansia muciniphila
Alistipes
Anaerostipes hadrus
Aphantopus hyperantus
Arabia massiliensis
Bacteroides
Blautia
Burkholderiales
Butyricimonas faecalis
Coprococcus catus
Danio kyathit
Desulfovibrio fairfieldensis
Dysosmobacter welbionis
Eikenella corrodens
Enterocloster clostridioformis
Erithacus rubecula
Erysipelatoclostridium ramosum
Escherichia coli
Faecalibacterium prausnitzii
Flavonifractor plautii
Lachnospiraceae
Lactobacillus rennini
Megamonas funiformis
Parabacteroides distasonis
Paraprevotella xylaniphila
Phocaeicola
Poecilia reticulate
Porphyromonas crevioricanis
Prevotella
Roseburia intestinalis
Selenomonas sp. oral taxon 136
Spirometra erinaceieuropaei

Streptococcus
Veillonella
Victivallales
Unknown