

Two distinct patterns of *Clostridium difficile* diversity across Europe indicates contrasting routes of spread: Supplementary Material

Supplementary methods

Sequencing

Isolates from the 10 most prevalent toxigenic ribotypes underwent whole-genome sequencing. DNA was extracted from subculture of a single colony and sequenced using Illumina HiSeq2500, generating 100 base-pair paired-end reads. Sequence data were processed as previously,^{1,2} mapping reads to the *C. difficile* 630 reference genome (AM180355.1), except ribotype-027 and ribotype-078 isolates whose reads were mapped to CD196 (NC_013315.1) and M120 (FN665653.1) respectively.

Sequences were compared using single nucleotide polymorphisms, SNPs, obtaining differences between sequences from maximum likelihood phylogenies constructed from mapped read data using PhyML version 3.1³ (with generalized time-reversible substitution model and “BEST” tree topology search algorithm), corrected for recombination using ClonalFrameML version 1.25⁴ (with default settings). Sequence reads were also assembled *de novo* with Velvet,⁵ using VelvetOptimiser. Antimicrobial resistance determinants for fluoroquinolones, tetracycline, clindamycin/macrolides, aminoglycosides, and fidaxomicin were identified from mapped data (*gyrA*, *gyrB*, *rpoB*, *rpoC*, CD22120 point mutations/truncation) and using BLAST searches of *de novo* assemblies (*tet*, *ermB*, *aphA1*, and AAC(6′)-APH(2′) genes [details in table S1]). Allowing gene matches split across two or more contigs, genes were considered present where nucleotide identity was >80% along the entire gene length. Excepting AAC(6′)-APH(2′), all genes identified had ≥97% nucleotide identity, and only 6/8514 blast searches contained matches with <80% identity. *De novo* assemblies had to contain 3x10⁶ to 4.5x10⁶ bases within contigs ≥1k in length, otherwise the resistance genotype was defined as unknown. Table S2 provides sequencing quality metrics for reference-based mapping and *de novo* assemblies, by ribotype.

Phylogeographic analysis

BEAST version 2.4.3⁶ and BASTA version 2.3.0⁷ were used to reconstruct time-scaled phylogenies, and to infer the geographic location of each ancestral lineage. Input mapped sequence alignments had recombination removed using ClonalFrameML outputs. Three or more independent runs, each with ≥10 million iterations, were combined to determine the final trees, after discarding the first ≥1 million iterations of each run as burn-in, and ensuring similar convergence. A HKY substitution model was used. The mutation rate prior point estimate and uncertainty was set as previously estimated (log-normal prior, mean=-15.6, standard deviation=0.3).¹ Given the relatively small number of samples from each country, a single parameter was estimated for the within-country effective bacterial population size for all countries (log-normal prior, mean=4, standard deviation=8), and similarly a single

parameter was estimated for the symmetrical pairwise between-country migration rate, representing the transmission rate between countries (log-normal prior, mean=-8, standard deviation=2).

References

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- 8 Babakhani F, Seddon J, Sears P. Comparative microbiological studies of transcription inhibitors fidaxomicin and the rifamycins in *Clostridium difficile*. *Antimicrob Agents Chemother* 2014; **58**: 2934–7.
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- 10 Leeds JA, Sachdeva M, Mullin S, Barnes SW, Ruzin A. In vitro selection, via serial passage, of *Clostridium difficile* mutants with reduced susceptibility to fidaxomicin or vancomycin. *J Antimicrob Chemother* 2014; **69**: 41–4.
- 11 Dingle KE, Didelot X, Quan TP, *et al.* A role for tetracycline selection in the evolution of *Clostridium difficile* PCR-ribotype 078. *bioRxiv* 2018; 262352.

Supplementary figures

Ribotype 018/356

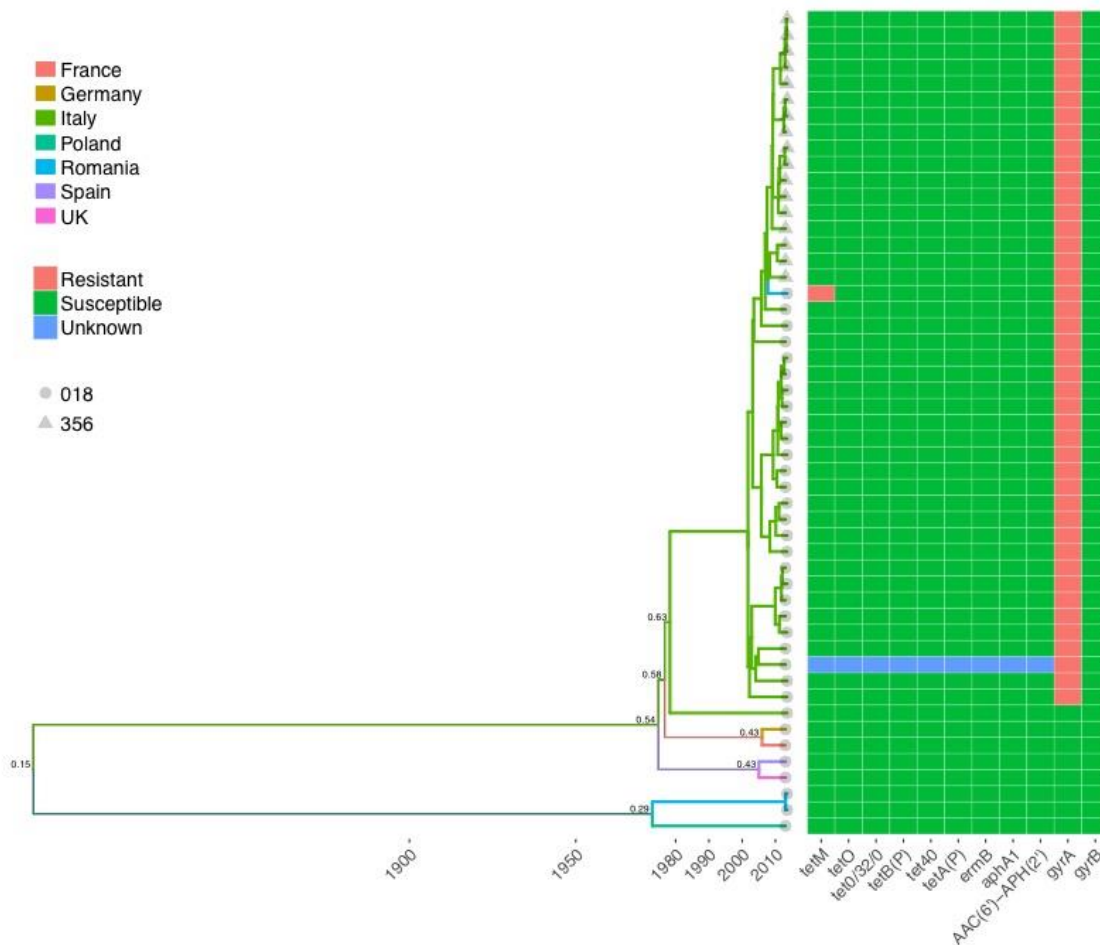


Figure S1. Combined ribotype-018 and ribotype-356 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants. The tree tips are annotated with a circle (ribotype-018) or a triangle (ribotype-356).

Ribotype 001

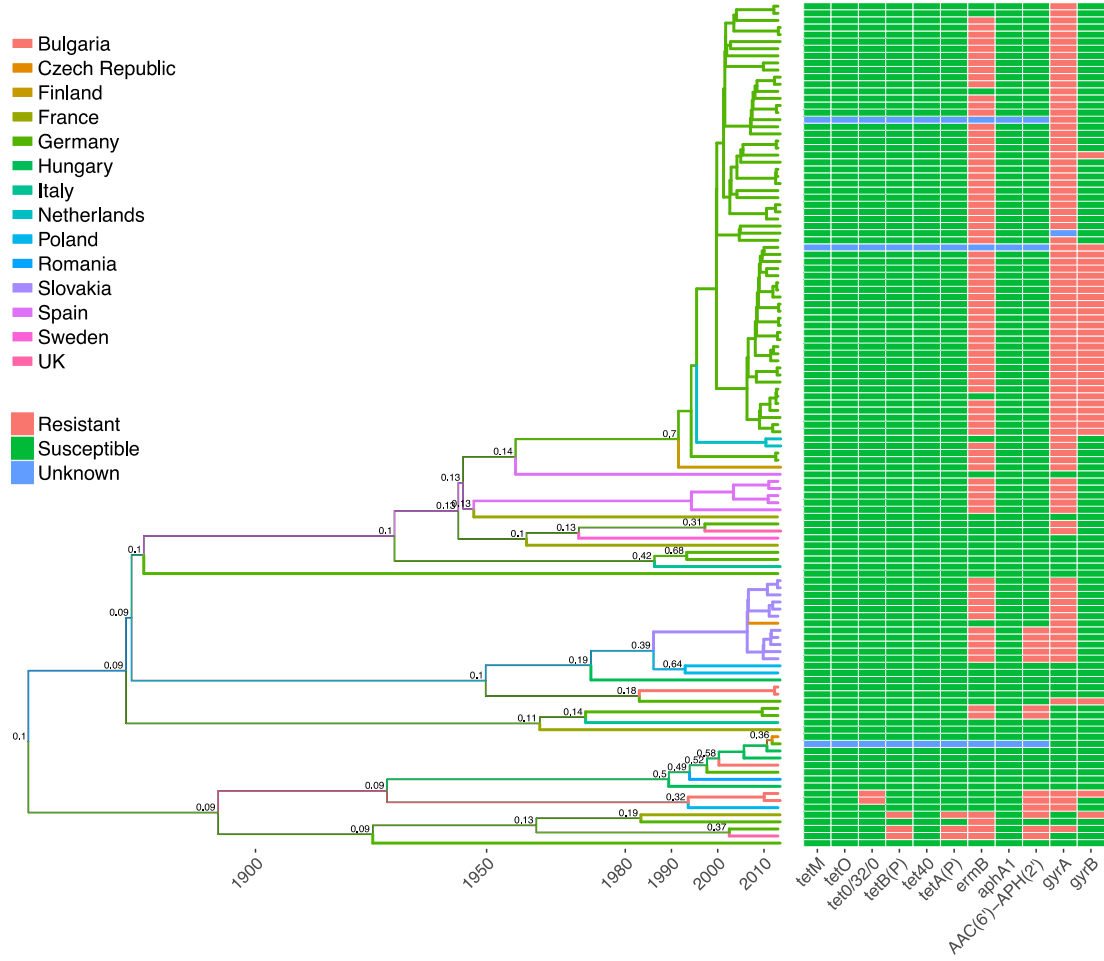


Figure S2. Ribotype-001 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants.

Ribotype 176

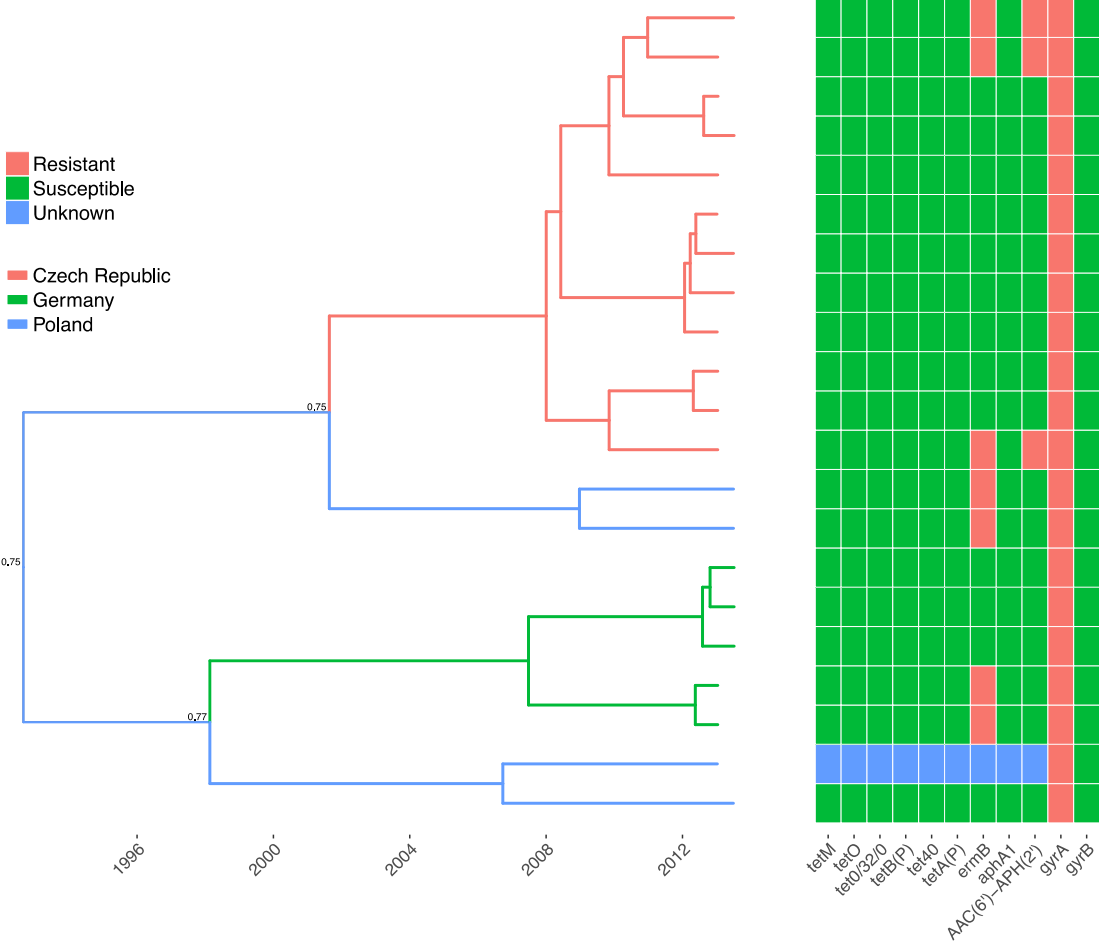


Figure S3. Ribotype-176 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants.

Ribotype 002

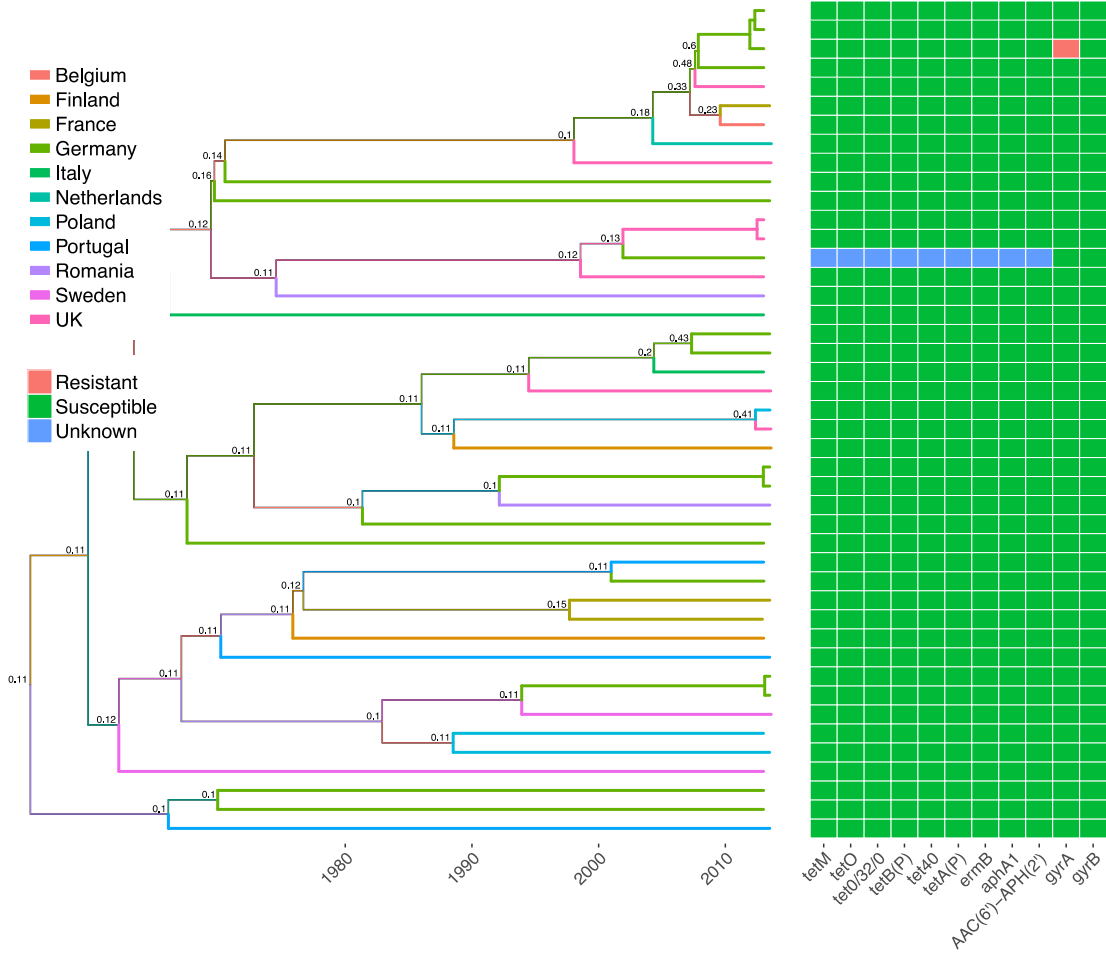


Figure S4. Ribotype-002 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants

Ribotype 014

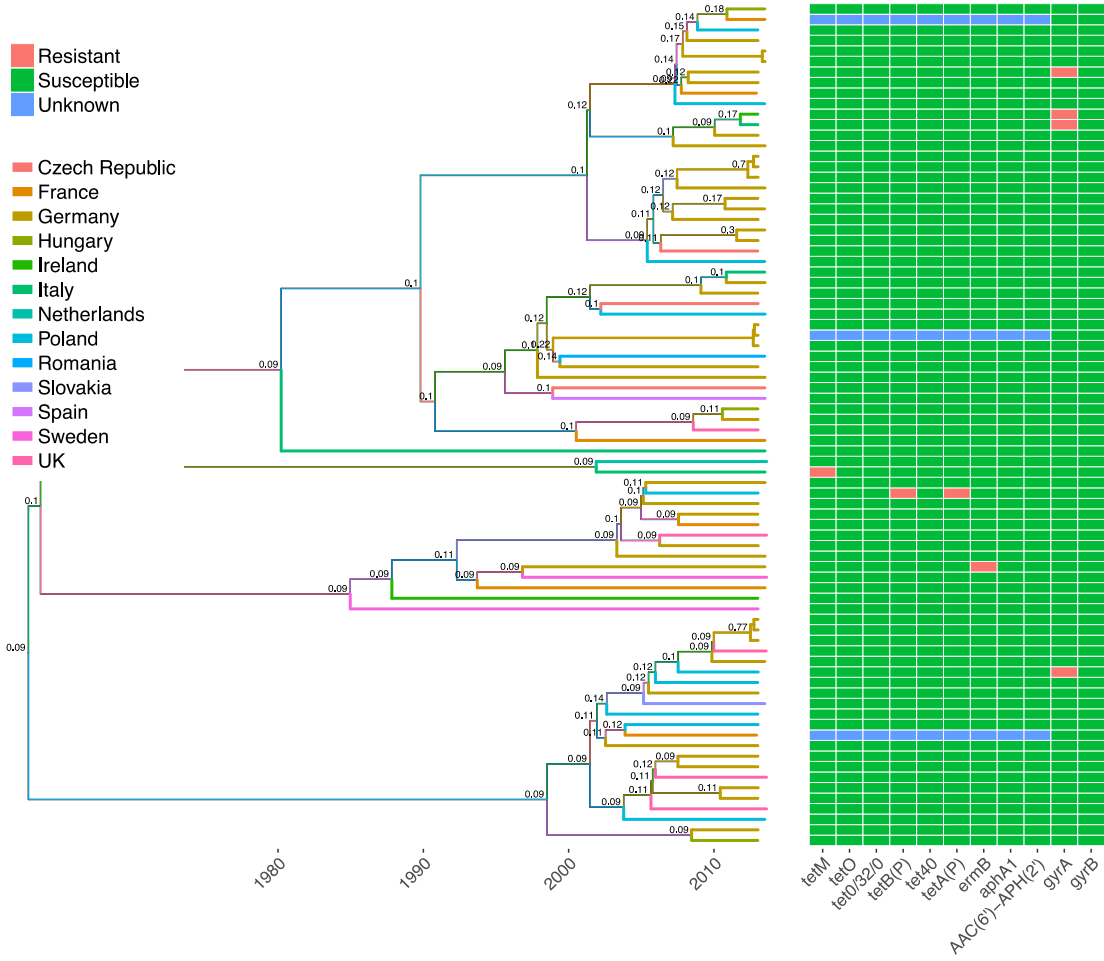


Figure S5. Ribotype-014 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants.

Ribotype 015

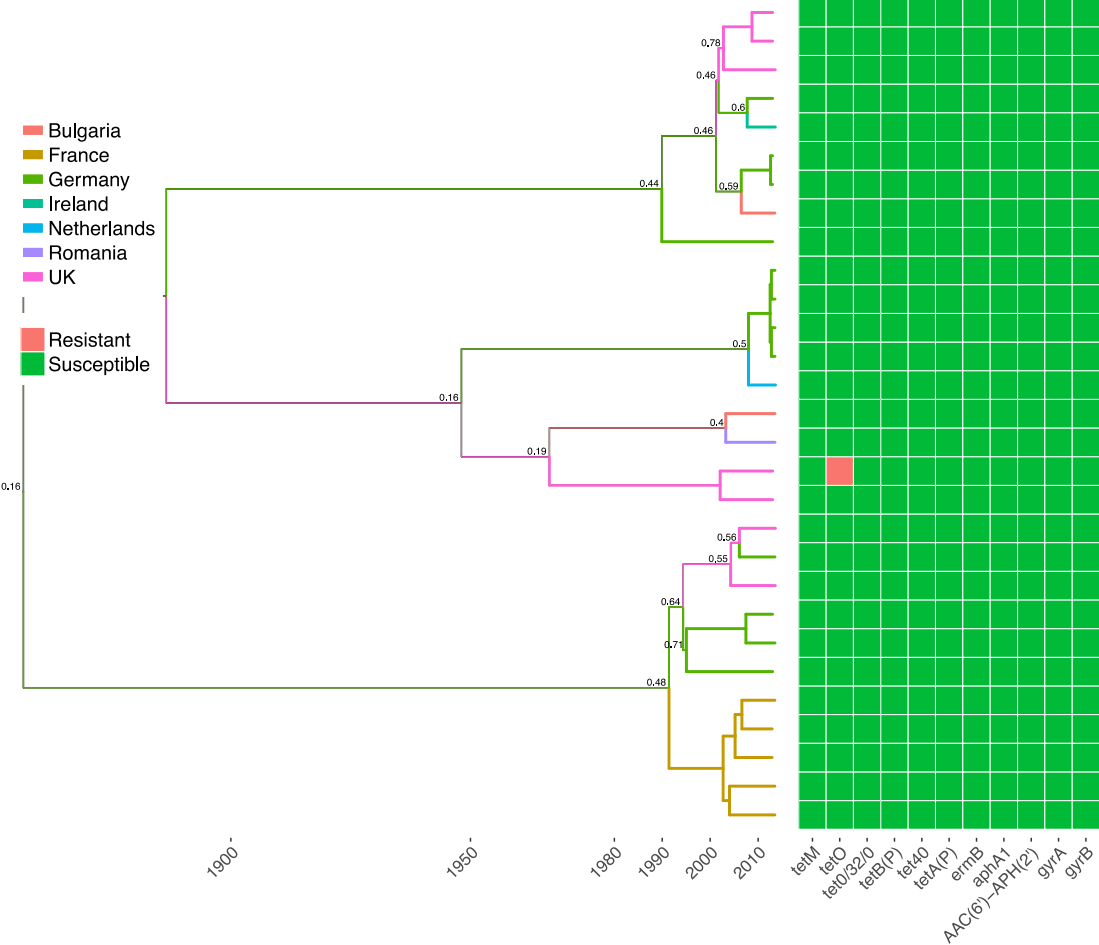


Figure S6. Ribotype-015 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants.

Ribotype 020

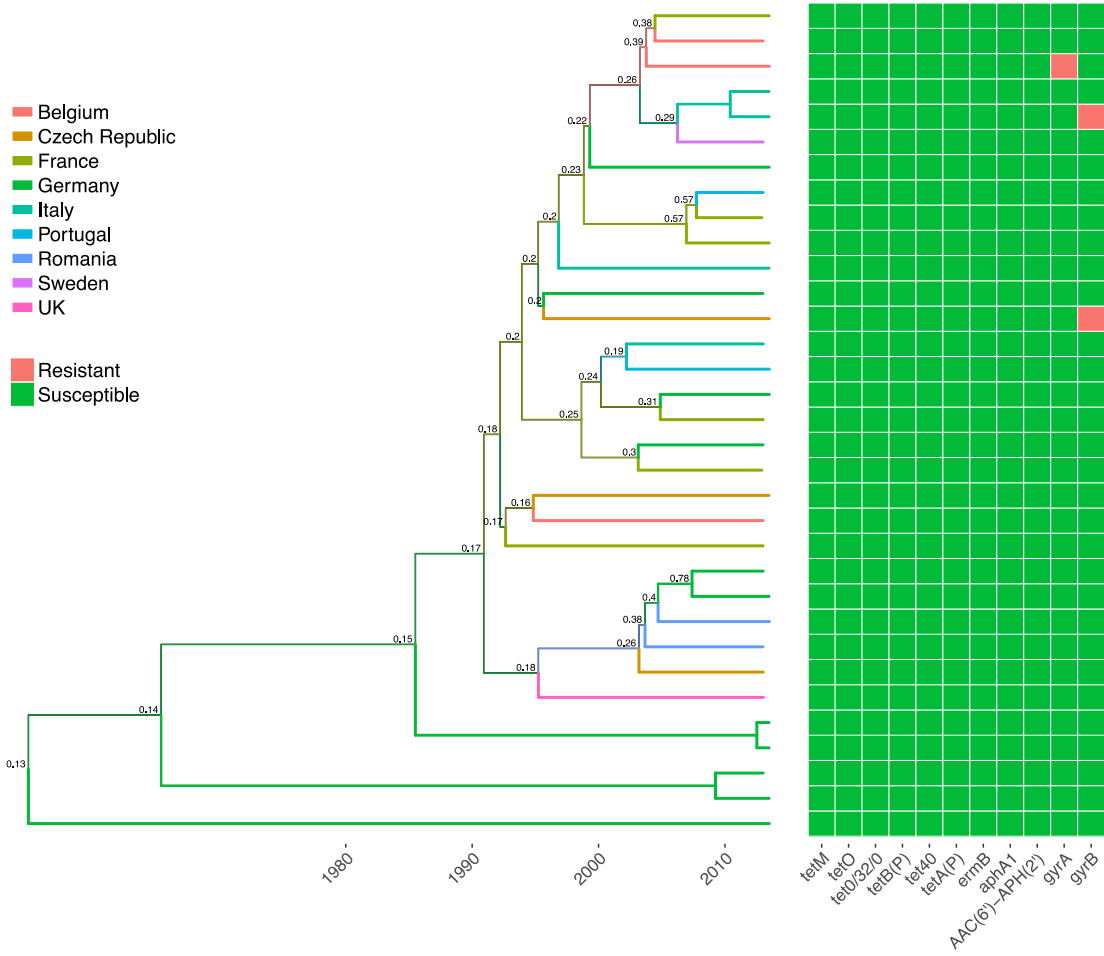


Figure S7. Ribotype-020 phylogeny with ancestral country reconstruction and antimicrobial resistance determinants.

Supplementary tables

Accessory gene	Reference sequence (GenBank)	Protein encoded	Antimicrobial resistance phenotype (predicted)
<i>tetM</i>	NG_048243.1	Ribosomal Protection Protein	Tetracycline
<i>tetO</i>	AY394561.1	Ribosomal Protection Protein	Tetracycline
<i>tetW</i>	FR838948.1	Ribosomal Protection Protein	Tetracycline
<i>tet0/32/0</i>	AJ295238.3	Ribosomal Protection Protein	Tetracycline
<i>tetB(P)</i>	NG_048319.1	Ribosomal Protection Protein	Tetracycline
<i>tet40</i>	JQ280445.2	Efflux pump	Tetracycline
<i>tetA(P)</i>	AB054980.1	Efflux pump	Tetracycline
<i>tetL</i>	NG_048203.1	Efflux pump	Tetracycline
<i>ermB</i>	HG002387.1	rRNA adenine N-6-methyltransferase	Macrolide-Lincosamide-Streptogramin B (MLS _B) antibiotics including clindamycin
<i>aphA1</i>	M26832.1	Aminoglycoside 3'-phosphotransferase	Aminoglycoside (streptomycin)
AAC(6')-APH(2')	M13771.1	6'-N-acetyltransferase and 2"-O-phosphotransferase activities, bifunctional	Most clinically important aminoglycosides

Housekeeping gene	Non-synonymous substitution	Protein Modified	Antimicrobial Resistance Phenotype
<i>gyrA</i>	T82I	DNA gyrase subunit A	Fluoroquinolones
<i>gyrB</i>	D426N	DNA gyrase subunit B	Fluoroquinolones
<i>rpoB</i>	V1143F, V1143G, V1143L, Q1074K	RNA polymerase subunit β	Fidaxomicin ^{8,9}
<i>rpoC</i>	D237Y	RNA polymerase subunit β'	Fidaxomicin ⁸
CD22120	Truncation	<i>marR</i> homolog	Fidaxomicin ¹⁰

Table S1. Antimicrobial resistance determinants searched for. Based on resistance determinants previously described by Dingle *et al*¹¹.

Ribotype	Mapping		De novo assembly	
	Proportion of reference genome called	N50	Number of contigs	Megabases of sequence in contigs >1k long
001	0.835 (0.827 - 0.845)	6627 (4600 - 17802)	1244 (585 - 1624)	4.02 (3.87 - 4.13)
002	0.829 (0.821 - 0.842)	8071 (4858 - 28280)	944 (417 - 1496)	3.96 (3.81 - 4.09)
014	0.839 (0.828 - 0.851)	6338 (4938 - 11262)	1261 (752 - 1476)	3.95 (3.81 - 4.07)
015	0.840 (0.831 - 0.846)	7134 (5071 - 11040)	1197 (837 - 1497)	4.16 (4.02 - 4.26)
018	0.838 (0.822 - 0.848)	7246 (5125 - 22680)	1088 (526 - 1507)	4.08 (3.95 - 4.19)
020	0.843 (0.833 - 0.856)	6534 (4931 - 25800)	1168 (554 - 1502)	3.97 (3.86 - 4.09)
027	0.923 (0.911 - 0.934)	7461 (5151 - 20464)	1080 (531 - 1460)	3.94 (3.81 - 4.06)
078	0.928 (0.915 - 0.938)	6198 (4068 - 17192)	1140 (352 - 1568)	3.76 (3.57 - 3.90)
106	0.828 (0.828 - 0.828)	4736 (4736 - 4736)	1610 (1610 - 1610)	3.78 (3.78 - 3.78)
176	0.832 (0.829 - 0.836)	8008 (5337 - 12079)	905 (641 - 1039)	3.98 (3.86 - 4.03)
356	0.841 (0.831 - 0.849)	6635 (5106 - 46375)	1123 (327 - 1529)	4.08 (3.90 - 4.12)

Table S2. Sequencing quality metrics, median (interquartile-range) by ribotype. N50 is a weighted median statistic such that 50% of the entire assembly is contained in contigs equal to or larger in length than this value. Samples were mapped to the *C. difficile* 630 reference genome (AM180355.1), with the exception of ribotype-027 which was mapped to the CD196 reference genome (NC_013315.1) and ribotype-078 which was mapped to M120 (FN665653.1).

Identifier	Ribotype	Collection date	Country
4b7b0a87-6283-4b0c-8a06-6baabc112bd7	001	2013-01-08	Bulgaria
14503ff2-51cf-4488-8a74-58d8c34e69c7	001	2013-01-08	Bulgaria
84c50328-4863-4f97-a509-6505ed55de2c	001	2013-01-10	Bulgaria
f879c402-aa4c-428a-a173-82e5cd256277	001	2013-01-15	Bulgaria
ca35c6c7-ad70-4f2b-885d-ddd19f69055b	001	2013-07-12	Bulgaria
e43a403a-248b-49ca-926a-ab8688fe2df6	001	2013-01-14	Czech Republic
cf1aedbd-f655-40f2-8bb0-80a0eee9a2d0	001	2013-01-15	Czech Republic
59339567-82ee-414b-ab8d-139596c2e1bd	001	2013-08-10	Finland
2b8ef645-edb6-4892-a6fc-7277573aedaa	001	2012-12-03	France
d4717931-a4d9-4d9f-8263-0ee0bf7592cc	001	2012-12-06	France
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a79e1932-f0f2-4898-b797-57461d7995da	001	2013-07-03	France
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57a5f566-116d-48b3-9d34-1ac99b3482a6	078	2013-07-10	Germany
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c6755f7e-697c-422a-ab7f-b23689f3d00b	078	2013-01-06	Ireland
4097a98a-3bbf-42a1-8d1b-0734ef75bcc2	078	2013-08-05	Ireland
196696b4-e630-41e9-bb94-c4f43a4c5cb0	078	2013-01-06	Italy
3597f2ef-c8d5-4afb-b21e-915216c8d136	078	2013-01-07	Italy
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c10dbbb6-56f4-4afa-9519-b5bd16d64b20	078	2013-01-08	Portugal
b263a735-6b21-4b70-8ec8-c7721b00ed6f	078	2013-01-17	Portugal
cc8c1189-f7be-4664-b6c8-dbe6818e0579	078	2013-07-09	Portugal
7cbad540-efbb-4158-9227-5641bc9194ea	078	2013-07-09	Spain
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1269f0f4-583f-4239-8e70-f7640bd350dc	078	2013-08-05	UK
66108826-7a8c-4f03-8da1-3b6cd3cde48e	078	2013-08-05	UK
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5b728dd2-6fae-40cb-9a0d-103c02f335fc	176	2013-01-09	Czech Republic
735dedf8-5856-4b0b-8148-4b1705b7a4f3	176	2013-01-14	Czech Republic
23bed506-bdef-4316-ae8a-969350be4d66	176	2013-01-14	Czech Republic
799cf0f8-bc9c-4284-8327-be095c3ae8ac	176	2013-01-14	Czech Republic
26ace4fe-313c-4398-87c2-a9c5716823e8	176	2013-01-14	Czech Republic
0b7a0a5b-9b3b-4ba2-b1a4-5aabc7d334c8	176	2013-01-15	Czech Republic
3b35d73c-db0d-4283-a8e8-7dbb0ca7835f	176	2013-01-18	Czech Republic
72711a33-d4a1-4b8a-913c-cd3e427858b6	176	2013-07-01	Czech Republic
7ac75243-28c0-4cf6-8fd9-889354f2b857	176	2013-07-02	Czech Republic
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ce8fe3f1-c679-4722-b775-d586d80ae9a6	176	2013-07-09	Czech Republic
75277d56-150b-4bf1-8704-58bce5db8a49	176	2013-01-14	Germany
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2397afdb-7286-4d87-8a6a-e6032c0dfab5	176	2013-07-09	Germany
f80e655b-e858-4a66-9bf4-3ed99ac9d401	176	2013-07-09	Germany
1d4509b9-ce9e-479c-9f34-c93e3771f0a5	176	2013-07-09	Germany
93a8808e-e0f2-4e67-8cf7-ee44985c72f9	176	2013-01-10	Poland
40408d06-ba39-4f3a-b6eb-bdbb967a1c45	176	2013-07-01	Poland
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87d95eb1-7794-4bd3-8b0d-45854e4859d8	356	2013-01-06	Italy
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57ecce95-9ba7-4e94-87b7-dfdf796fdabd	356	2013-01-08	Italy
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0c166457-d7d2-41aa-ac6f-8753856d3731	356	2013-01-15	Italy
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24f4e049-2bb6-40fa-8d45-6bf34521817a	356	2013-07-02	Italy
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2017f91e-9f2a-47b6-a247-a91beab10dcd	356	2013-07-09	Italy

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81c4d8ed-19b1-4926-947e-4ce95d3a35ce	356	2013-07-09	Italy

Table S3. Sequenced isolates.

