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Supplementary appendix 1

This appendix formed part of the original submission and has been peer reviewed. We post it as supplied by the authors.

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Supplementary Methods

Additional information regarding the Euro-GASP

The European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) is coordinated by the European Centre for Disease Prevention and Control (ECDC) and it has since 2009 been implemented by the UK Health Security Agency (UKHSA), London, United Kingdom and the WHO Collaborating Centre for Gonorrhoea and Other STIs, Örebro University Hospital, Örebro, Sweden (WHO CC).[1-7] In the Euro-GASP 2020 survey, 3291 gonococcal isolates linked to patient metadata (demographic, epidemiological and clinical data) were collected in 23 European Economic Area (EEA; 27 European Union countries plus Iceland, Liechtenstein and Norway) countries.[2] In the present Euro-GASP 2020 genomic study, 1932/3291 (58·7%) consecutive isolates from 21 EEA countries were examined using whole genome sequencing (WGS) in conjunction with antimicrobial resistance (AMR) data and patient metadata (Table 1, Supplementary Figure 1). Isolates from 16 countries (76·2%) were AMR tested decentralised (in their country), while isolates from five countries (23·8%) were tested at the UKHSA or WHO CC. Minimum inhibitory concentration (MIC) gradient strip tests (i.e., Etest for ceftriaxone, cefixime, azithromycin) and MIC gradient strip tests or agar dilution breakpoint technique (for ciprofloxacin) were performed as earlier described.[2] As defined by the European Committee on Antimicrobial Susceptibility testing (EUCAST), S (susceptibility), I (susceptibility, increased exposure) and R (resistance) are used to categorise isolates in Euro-GASP. The current EUCAST clinical SIR breakpoints (v14.0) were used for ceftriaxone (S≤0.125 mg/L, R>0.125 mg/L), cefixime (S≤0.125 mg/L, R>0.125 mg/L), and ciprofloxacin (S≤0.03 mg/L, R>0.06 mg/L), and the EUCAST epidemiological cut-off (ECOFF) value of MIC>1 mg/L for azithromycin (referred to as resistance in the manuscript), because clinical breakpoints are lacking for azithromycin (https://www.eucast.org/clinical_breakpoints). Several quality controls were applied to quality-assure the centralised and decentralised AMR testing, as previously described.[2,7] Repeated MIC determination and/or WGS was performed for some isolates with discrepancies between phenotype and genotype. Euro-GASP aims to collect ≥100 isolates per participating country and year, so countries with low gonorrhoea incidence or those who rarely perform gonococcal culture also include isolates from outside the official temporal window for collection (1st September-30th November), see Supplementary Figure 1. In countries where 100 isolates represent <10% of the annual number of reported gonorrhoea cases, 200 isolates are recommended to be included with the aim to provide a more representative sample.[2] In the

present Euro-GASP 2020 WGS study, 150 consecutive isolates and 75 consecutive isolates were selected from Euro-GASP countries requested to annually provide 200 isolates and 100 isolates, respectively.[2] The Euro-GASP has been shown to adequately reflect the AMR situation for gonococci in the EEA.[1]

Table 1 and Supplementary Figure 1 summarise the number of isolates included in the Euro-GASP 2020 survey and the number of those included in the present genomic study.

In Euro-GASP, isolates should be collected from consecutive patients, ideally representing different patient groups and geographical regions within the countries. When >1 viable gonococcal isolate is available from a patient during the same gonorrhoea episode, a sampling hierarchy is applied. For males, isolates are prioritized in the following order: 1) from the pharynx, 2) anorectum, 3) urethra, and 4) other. For females, isolates are prioritized from: 1) the pharynx, 2) cervix, 3) anogenital tract (high vaginal, rectal or urethral), and 4) other.

Countries submit the AMR data of the gonococcal isolates and the metadata of the patients to The European Surveillance System (TESSy) at ECDC.[2] Epidemiological data include, where available, the sampling date, anatomical site of sample, sex, age, sexual orientation, previous gonorrhoea diagnosis, concurrent STIs, place of residence, type of clinic, HIV status, and probable country of infection. The gonorrhoea patients in the Euro-GASP 2020 WGS study adequately represented all the Euro-GASP 2020 gonorrhoea patients.[2] No patient identifiable information was available in the present Euro-GASP 2020 WGS study, so separate ethical approval was not required.

All gonococcal isolates were cultured and preserved as part of the routine diagnostics (standard care), and isolates or data were submitted to the Euro-GASP surveillance study with no patient identification information, separate ethical approval was therefore not required.

DNA extraction and whole genome sequencing

Frozen gonococcal isolates (n=1302) from fifteen countries were sent to the UKHSA or WHO CC. Genomic DNA was extracted from pure culture (after subculturing of a single colony) using the QIAsymphony DSP Virus/Pathogen Kit (Qiagen, Hilden, Germany) on the QIAsymphony (Qiagen) instrument with minor modifications. DNA extractions were quality controlled for concentration using Qubit Flex fluorometer (Thermofisher, Waltham, MA, USA) and for RNA/protein contamination as well as overall quality using the 4200 Tapestation System (Agilent, Santa Clara, CA, USA) with Genomic DNA kit to achieve DNA Integrity Number (DIN) ≥8. Centralised WGS was performed at the WHO CC for 15/21 (71·4%)

countries and 6/21 (28·6%) countries performed decentralised WGS using Illumina paired-end technology, according to quality criteria, and uploaded fastq files to the WHO CC. DNA libraries were prepared using the Illumina DNA Prep (Illumina, San Diego, CA, USA) on a 96-plex automated Microlab STAR platform (Hamilton, Reno, NV, USA). Libraries were normalized as previously described[8] and sequenced using NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) on Nextseq 550 (Illumina) with read length of 149 bp. Fastq files were obtained using bcl2fastq (v2.20.0.422) with default settings and --no-lane-splitting, generating 149 bp paired-end reads with ideally >70x in depth. Decentralised sequencing of isolates (n=671) was performed in six countries using paired-end sequencing on the Illumina platform with >75% average Q30 and expected average depth of ≥70x.

Sequencing quality control and whole genome assembly

In total, data from 1973 isolates were available for analysis. Raw sequences obtained from WGS were quality checked (QC), assembled and mapped using the nullarbor "Reads to Reports" pipeline v2.0.20191013 (https://github.com/tseemann/nullarbor), which processes batches of bacterial raw paired-end short-read data. The pipeline removed adaptors, low quality bases and reads with Trimmomatic v0.39[9] by cutting bases from the start and end of the reads if they are below a Phred score of 30 (Q30). SPAdes v3.14[10] was used as the genome assembler with the --careful option in order to reduce the number of mismatches and short indels. Final assemblies were annotated using Prokka (v1.14.6) and pan-genome analysis was obtained using Roary (v3.13.0). The final steps in the pipeline included mapping all reads to FA1090 (Genbank: AE004969.1) and calling variants using snippy (v4.4.3) prior to construction of phylogenomic tree using iqtree2 (v2.0.7). A final report including all isolates was generated using nullarbor-report.pl (v2.0.20191013) and this report was used for the QC, data that did not clear the initial assessment by the nullarbor pipeline was excluded. The initial assessment included read-depth of ≥25x and ≥65% reads classified as *Neisseria gonorrhoeae,* which excluded 12 isolates. Sequence data classified 65-85% as *N. gonorrhoeae* was subject for KrakenTools (https://github.com/jenniferlu717/KrakenTools) and only taxon id 485 (*N. gonorrhoeae*) classified reads were extracted for downstream analysis. Secondary assessment included assembly and annotation, where the average contig number and assembly size was calculated and all isolates with 1.5 times the average number(s) were flagged for third assessment. The third assessment used the number of annotated genes (pan-genome analysis) and the proportion of mapped reads, any isolate with >2500 annotated genes and/or <70% mapped reads to the

reference, respectively, was excluded (n=9). Finally, all heterozygous sites were evaluated and isolates within the top 99% quantile was considered cross-contaminated and excluded (n=20). Final assemblies were uploaded into Pathogenwatch.[11] A second pipeline in CLC Genomics Workbench v22.0.1 (Qiagen) was used to detect the variant frequency within the 4-ploidy regions of the 23S rRNA gene as well as *mtrRCDE* characterization according to an in-house *mtr* database. All novel *mtr* types were curated and added to the *in house* database.[12] *N. gonorrhoeae* numbering was used for the 23S rRNA gene mutations.

Genomic data from previous Euro-GASP WGS studies (PRJEB9227 and PRJEB34068) were acquired and analysed as above with the Euro-GASP 2020 data for comparison. The data used consisted of 1053 and 2374 genomes from 2013[4] and 2018,[5] respectively.

The raw data for Euro-GASP 2020 is available through European Nucleotide Archive (ENA) accession number PRJEB58139. Furthermore, all WGS sequences of the isolates are uploaded and made available linked to the accession numbers and AMR profiles, country, and year in Pathogenwatch (https://pathogen.watch/collection/cqnydbxm603s-eurogasp-2020).

Detection of genetic antimicrobial resistance (AMR) determinants and molecular typing

Molecular typing of isolates included MLST (https://pubmlst.org/) and *N. gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR) (https://ngstar.canada.ca/) sequence types (STs) and was obtained from the Pathogenwatch (https://pathogen.watch/)[11] characterisation when uploading the SPAdes v3.14[10] assemblies. The final assemblies were also analysed using mlst v2.19.0 (https://github.com/tseemann/mlst) and pyngSTar (https://github.com/leosanbu/pyngSTar) to obtain new allele sequences in batch for submission and designation to PubMLST (https://pubmlst.org/) and NG-STAR (https://ngstar.canada.ca/) databases, respectively. In total, 25 new MLST and 114 new NG-STAR STs were found. Furthermore, the NG-STAR database consisting of 4623 STs was downloaded (26th of June 2022) and the unique allelic profiles were clustered using global optimal eBURST analysis in the PHYLOViZ toolkit v2.0[13] to designate the STs into 469 NG-STAR clonal complexes (CCs) as previously described.[14] The database was used as input to pyngSTar and to assign NG-STAR CCs to all isolates in the present study. The original *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) database (www.ng-mast.net) is no longer available and, accordingly, the NG-MAST data from Euro-GASP 2013[4] and Euro-GASP 2018[5] could not be appropriately compared to the Euro-GASP 2020 dataset.

The AMR determinants for ESCs, azithromycin, and ciprofloxacin were characterized using a customized CLC Genomics Workbench v22.0.1 (Qiagen) workflow as previously described.[12] Furthermore, Pathogenwatch[11] was used to characterize AMR determinants when suboptimal assembly was obtained by the CLC workflow.

Multiple sequence alignment (MSA) obtained from the nullarbor pipeline was used to construct the phylogenomic tree using iqtree2 v2.0.7[15] based on 56,199 informative sites. The best-fit model out of 286 DNA models tested, was general time reversible with discrete gamma model and ascertainment bias correction. Gubbins was used to remove recombinant regions.[16] Phylogeography and related metadata were visualized in Microreact.[17] Lineages were defined as previously described[18] using an updated collection of 33900 publicly available genomes. Briefly, ENA was queried for taxon 485 (14th Aug 2022) and raw data from bioprojects using next generation DNA sequencing with >10 isolates and not including other species was downloaded. All data was assembled using velvet, assembled genomes were typed according to a predefined core genome MLST of 1700 genes and an accessory genome of 362 genes[18] using Seqsphere v8.5.1 (Ridom GmbH). A neighbour joining tree was constructed based on the allele designations for each isolate for the 2062 genes. Treecluster[19] was used with root_dist method and a threshold of 0.045 to define lineages. Moreover, full *mtrRCDE* gene sequences were extracted for isolates which did not have a 100% hit in the in-house CLC Genomics Workbench database. All novel *mtrRCDE* loci were identified, curated for mosaicism and novel sequences were added to the *in house* database.[12] In total, there were 8, 34 and 19 novel *mtrC*, *mtrD*, and *mtrE* sequences, respectively.

Statistical analyses

Univariate statistical associations among the MLST STs, NG-STAR STs and NG-STAR CCs (with >10 isolates) and epidemiological data (age category, sex, sexual orientation, and site of infection) were calculated using odds ratio with the *oddsratio* function of the *epitools* R package, including 95% confidence intervals. To do so, a contingency table was calculated for the combination of each of the STs and the mentioned epidemiological variables. By default, Wald's test was used as method for the calculation of odds ratio except when there were numbers below 5, when Fisher's exact test was used. The base reference used for each of the epidemiological variables used was "≥45 years" for age category, "F" (female) for sex, "HETERO" (heterosexual) for sexual orientation and "GEN" (genital) for site of infection. Multivariate logistic regression models were also built to study these associations using the *glm*

(Generalized Linear Model) function from the *stats* package in R.[20] For this, each isolate was classified as whether they were part of an ST and this information was used to build the binary outcome variable. The four variables under study (age category, sex, transmission and site of infection) were used as predictor variables of the model. The Z statistic was used to calculate the p-values for the coefficients of these variables to assess the significance of the contribution of each variable to the model and thus, the significance of each variable to each ST. The p-values in this case were not corrected for multiple testing because they were obtained from multivariate tests.

For SIR antimicrobial resistance data, the odds ratio was calculated similarly: for each ST, a 2-by-2 contingency table was built considering the number of susceptible and resistant isolates in the dataset for the target ST compared to the rest of the isolates in the collection. Wald's or Fisher's exact test was used depending on whether any of the numbers in the table was below 5, as described above. "S" (susceptible) was used as base reference. As the SIR data are only reported as resistant or intermediate resistant MIC values above a threshold (breakpoint or ECOFF), we applied logistic regression to calculate the association of the STs with changes in the MIC values within the same category (i.e. small increases in MIC that do not reach the resistant threshold).

P-values for the univariate association tests among sequence types and epidemiological variables or AMR data were corrected for multiple testing using False Discovery Rate (FDR) by using the *p.adjust* function in the *stats* package in R.[20] P-values<0·05 after FDR correction were considered statistically significant.

Tajima's neutrality test was performed using multiple sequence alignments for isolates collected from different countries in 2018 and 2020 with MEGA11 software[21] by calculating the number of segregating sites, nucleotide diversity, and Tajima's D.[22]

Supplementary Note 1

***mtr* mosaic sequences found in the Euro-GASP 2020 dataset**

Mosaic structures in the *mtr* operon have emerged as the most common cause of low-level azithromycin resistance.[5,11,18,23,24] The encoded MtrRCDE proteins have regions with high disorder outside the predicted membrane segments suggesting motility. Mosaicism is defined as having recombinations from closely related species in all such regions. We defined semi-mosaicism as mosaic structures in only one of these regions. MtrD semi-mosaics additionally harboured previously described MtrD amino acid alterations associated with increased MICs of azithromycin, i.e., in amino acids R714 and/or K823.[25,26]

In the Euro-GASP 2020 collection, 239/1932 isolates (12·4%) carried a mosaic *mtrR* promoter in four different variants. The majority of isolates had a *N. lactamica*-like mosaic *mtrR* promoter allele and variant 2[5,11,23,24] was the most common (n=230) followed by variant 4 (n=5) and variant 5 (n=3). One isolate carried *N. meningitidis*-like mosaic *mtrR* promoter variant 1. No isolate in this study had mosaic *mtrR* promoter variant 3.[5,11,23,24] Four variants of mosaic *mtrR* were found in 239 (12·4%) isolates and only four (0·2%) of these isolates lacked mutations in 23S rRNA or *mtrD* mosaic, but had mosaic/semi-mosaic *mtrC*. Three of the four isolates had a GC base pair deletion in *mtrC.* All these four isolates were azithromycin susceptible (MIC=<0·064-0·125 mg/L). The isolates were designated to 47 different *mtrC* variants, of which six were mosaic *mtrC* variant that were found in 238 (12·3%) isolates, the most common being variant I (n=204). Furthermore, 153 (7·9%) isolates hade *mtrC* semi-mosaic structures and variant S (n=123) was the most common. Thirty-one different variants of *mtrE* were found, of which six variants were mosaic and two semi-mosaic. Mosaic and semi-mosaic *mtrE* was found in 252 (13·0%) and 224 (11·6%) isolates, respectively. The most common mosaic and semi-mosaic *mtrE* variants were variant C (n=219) and variant D (n=223). Fifty-one different *mtrD* variants were found, of which 18 variants were mosaic and four semi-mosaic (Supplementary Figure 3, Supplementary Table 2, https://microreact.org/project/mtrd-alleles). Mosaic *mtrD* variants were found in 392 (20·3%) isolates, of which mosaic 2[5,11] (n=201) was the most common, followed by variant C (n=112) and variant 34 (n=29) (Supplementary Figure 2, Supplementary Table 2). Mosaic 2[5,11] was the only *mtrD* variant represented by more than one isolate (all of them also containing mosaic *mtrR* promoter and mosaic *mtrE*) and displaying an azithromycin MIC_{50} of >1 mg/L. Semi-mosaic *mtrD* variants were found in 190 (9·8%) isolates of which the most common was *mtrD* variant 13 (n=104) followed by variant 39 (n=84), variant 40 (n=1), and variant 41 (n=1).

Of the isolates that carried mosaic in all *mtr* genes, 104/189 (55·0%) were resistant to azithromycin. Of the 392 (20·3%) isolates carrying mosaic *mtrD* (18 different variants), 121/392 (30·9%) isolates showed resistance to azithromycin (104/392 (26·5%) of these isolates had *mtrD* mosaic $2^{5,11}$). All isolates with semi-mosaic *mtrD* variant 13 (n=104) also had semi-mosaic in *mtrE*, suggesting that a single recombination event had occurred involving segments of *mtrD* and *mtrE* due to the close proximity of the genes in the genome. Azithromycin resistance was found in 44/104 (42·3%) isolates with semi-mosaic structures in *mtrD* and *mtrE*. Furthermore, 5/84 (6·0%) semi-mosaic *mtrD* variant 39 were resistant to azithromycin while isolates belonging to semi-mosaic *mtrD* variants 40 and 41 were azithromycin susceptible. Seven (0·4%) isolates had only mosaic *mtrD* and these isolates had azithromycin MICs of 0·5-1 mg/L (4/7 *mtrD* variant 33 and 3/7 *mtrD* variant 50). Semi-mosaic structures in *mtrC* had no effect on azithromycin MICs.

In total, 98 *mtrD* variants are available in the in-house database, of which 51 variants were found in the Euro-GASP 2020 dataset (18 mosaic and four semi-mosaic; Supplementary Table 2). These *mtrD* mosaics are mainly related to *N. meningitidis* and *N. lactamica,* however two variants are closely related to *N. cinerea* (Supplementary Figure 3). The mosaic *mtrD* variants (in 392 Euro-GASP 2020 isolates) were all found in lineage A; including 233/249 (93·6%) isolates within NG-STAR CC63, 46/46 (100·0%) in CC213, and 34/242 (14·0%) in CC1387. Furthermore, semi-mosaic *mtrD* was also only found in lineage A; including all *mtrD* variant 13 belonging to the two closely related NG-STAR CC1031 (82/82, 100·0%) and CC1818 (22/68, 32·4%) (figure 2, Supplementary Figures 3 and 5). Similarly, isolates with semi-mosaic *mtrD* variant 39, 40, and 41 were closely related and mainly belonged to CC1615 (n=81) and CC1387 (n=2). The CC63 isolates were found in all participating countries besides Estonia (20/21 countries), while CC213 was found in 14/21 countries. The CC1031 (MLST ST9362) has emerged recently and was not previously found in Europe, isolates were mainly found in Sweden (n=33), Belgium (n=16), Germany (n=12), the Netherlands (n=11), and France (n=6) (Supplementary Figure 5).

Examining 33,900 isolates publicly available *N. gonorrhoeae* isolates showed a similar trend as Euro-GASP isolates with mosaic and semi-mosaic *mtrD* found in the more recently evolved strains in lineage A (Supplementary Figure 6), which depicts that the recombinant *mtrD* is a well-established trait in both the European and the global gonococcal population.

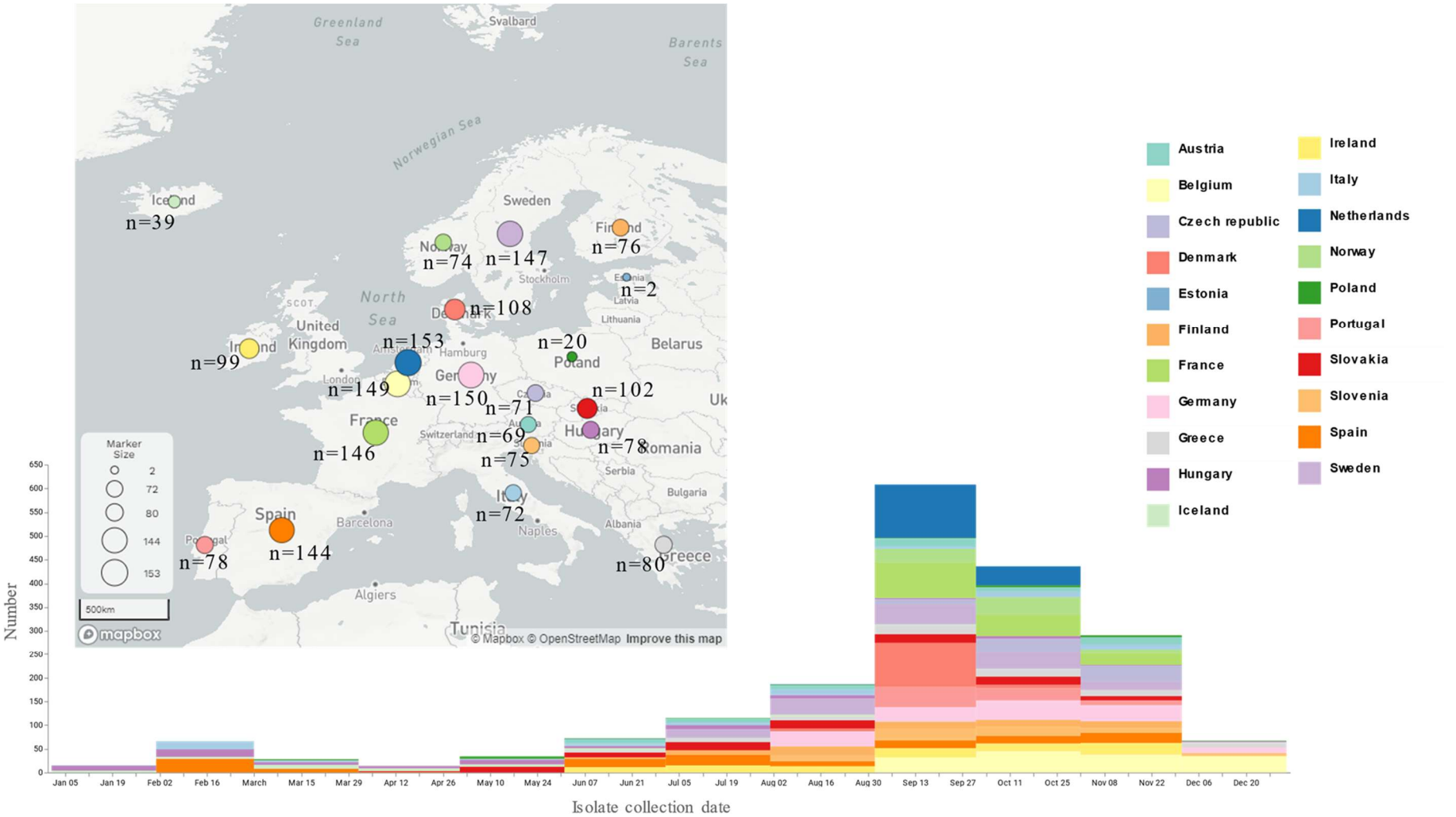
Supplementary Note 2

A decrease in the number of segregating sites, nucleotide diversity, and Tajima's D[22] in the gonococcal population from 2018 to 2020 elucidating a decreased genomic diversity and an increased clonality in the gonococcal population during the COVID-19 pandemic

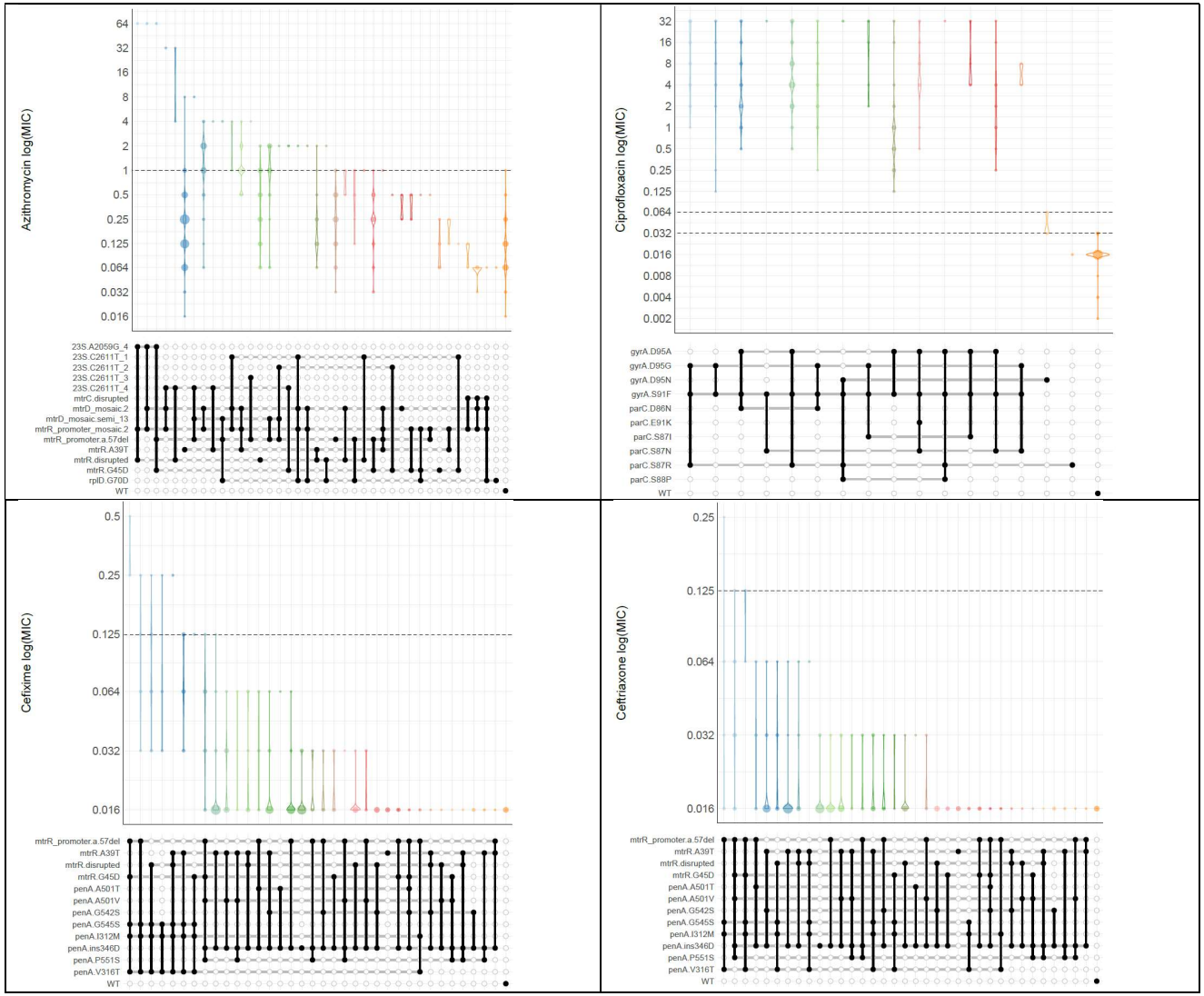
An increased clonality among *N. gonorrhoeae* isolates during the COVID-19 pandemic has been indicated in some countries.[27] To investigate and provide quantitative evidence regarding the impact COVID-19-associated national lockdowns and restrictions have had on the diversity of the *N. gonorrhoeae* population in Europe (nationally and regionally), the number of segregating sites and the nucleotide diversity were measured, and the Tajima's Neutrality Test (D)[22] used. Briefly, Tajima's D close to 0 is interpreted as a balanced evolution without selection pressure, a negative value is interpreted as a recent selective sweep in the population resulting in a decrease in diversity and potentially due to local spread of single gonococcal clones within a country under various levels of restrictions. In contrast, a positive value is interpreted as more variation within the gonococcal population. By combining Tajima's D and the COVID-19 Stringency Index (https://ourworldindata.org/covid-stringency-index), we explored whether various COVID-19-associated restrictions during 2020 may have impacted on the *N. gonorrhoeae* genomic diversity in EEA countries.

In 2020, examined isolates from all the EEA countries except Germany had a lower number of segregating sites and a decreased nucleotide diversity compared to 2018 (Supplementary Table 3). Similarly, a decrease in Tajima's D was observed in all countries that have had an average COVID-19 Stringency Index >50 during 2020 besides Slovenia (Supplementary Table 3), suggesting that the COVID-19-associated restrictions during 2020 had a substantial effect on the *N. gonorrhoeae* genomic diversity in nearly all EEA countries. Nevertheless, this is a very complex issue to provide appropriate evidence on and impact on the EEA gonococcal population of also additional factors, such as expansion or vanishing of specific AMR (or antimicrobial-susceptible) lineages, antimicrobial treatment, or other selective pressures, cannot be excluded.

Supplementary Figures

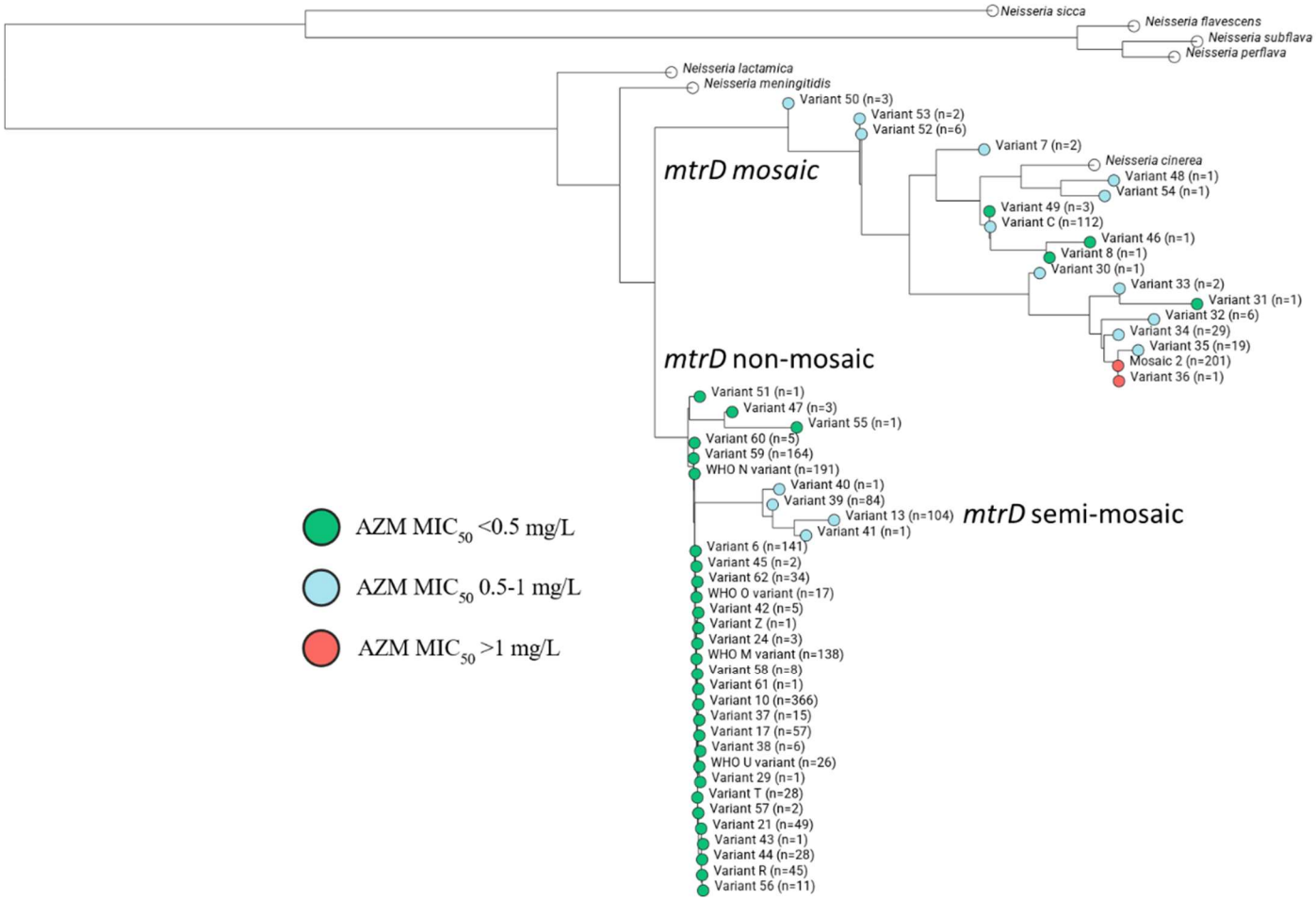


***Supplementary Figure 1: Neisseria gonorrhoeae* isolates (n=1932) examined in the Euro-GASP 2020 genomic survey, including 21 EEA countries.** The size of the circles in the map corresponds to the number of isolates in each country. Isolate collection dates are shown at the bottom. Euro-GASP=European Gonococcal Surveillance Programme. EEA=European Economic Area.

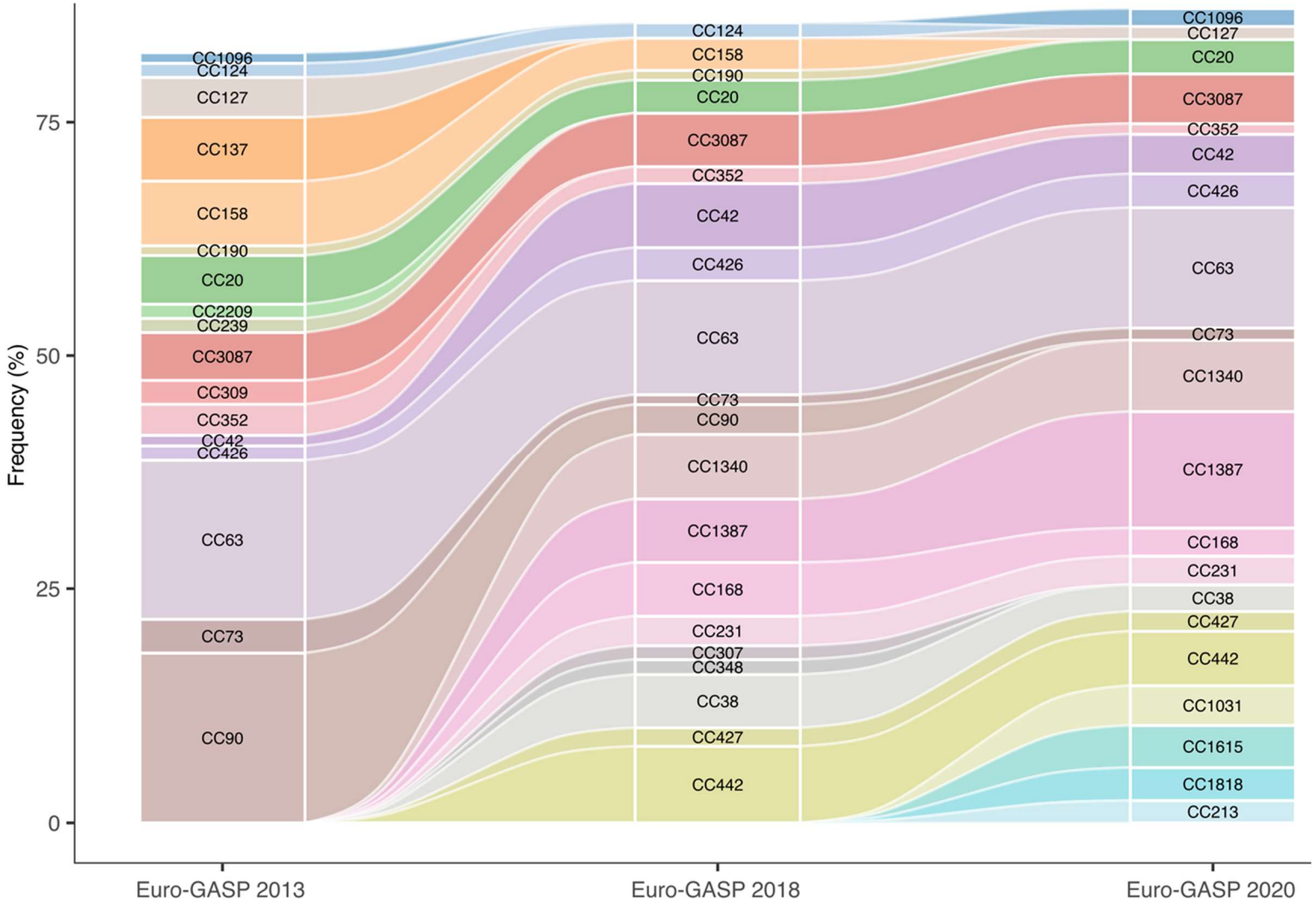


Supplementary Figure 2: **Concordance between phenotypic antimicrobial MICs (mg/L) and genetic determinants of AMR (single or in combination) in the Euro-GASP 2020 genomic survey, including 1932 *Neisseria gonorrhoeae* isolates from 21 EEA countries.** Y-axis

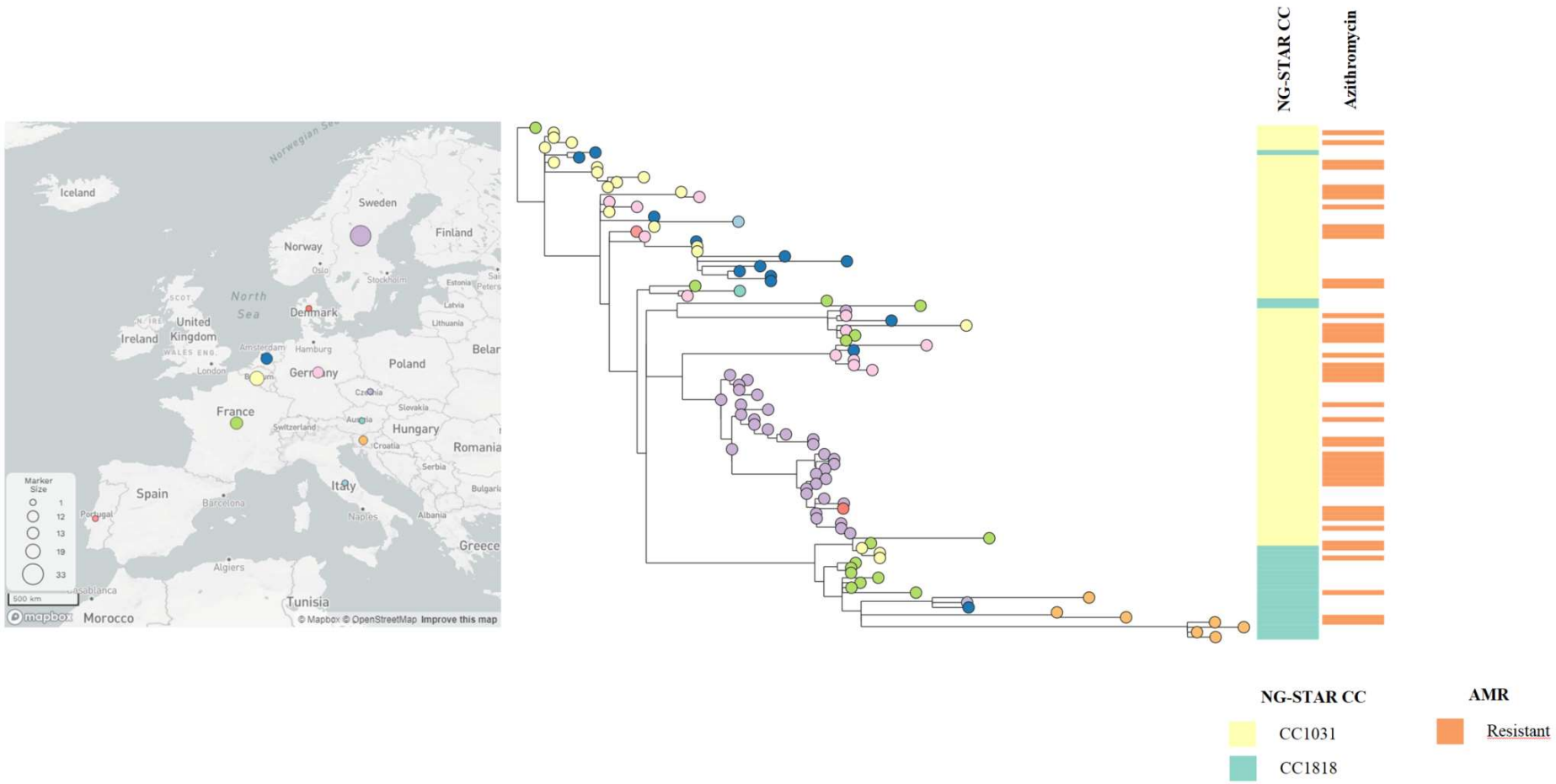
shows the MICs (coloured dots, sized based on number of isolates) and X-axis the AMR determinants (black dots linked with a solid black line to show their presence in combination). Violins are coloured to show different combinations of AMR determinants. Dashed horizontal lines represent the epidemiological cut-off for azithromycin and clinical resistance breakpoints for ciprofloxacin, cefixime and ceftriaxone established by the European Committee on Antimicrobial Susceptibility testing (EUCAST; www.eucast.org/clinical_breakpoints/). MIC=minimum inhibitory concentrations. AMR=antimicrobial resistance. Euro-GASP=European Gonococcal Surveillance Programme. EEA=European Economic Area.



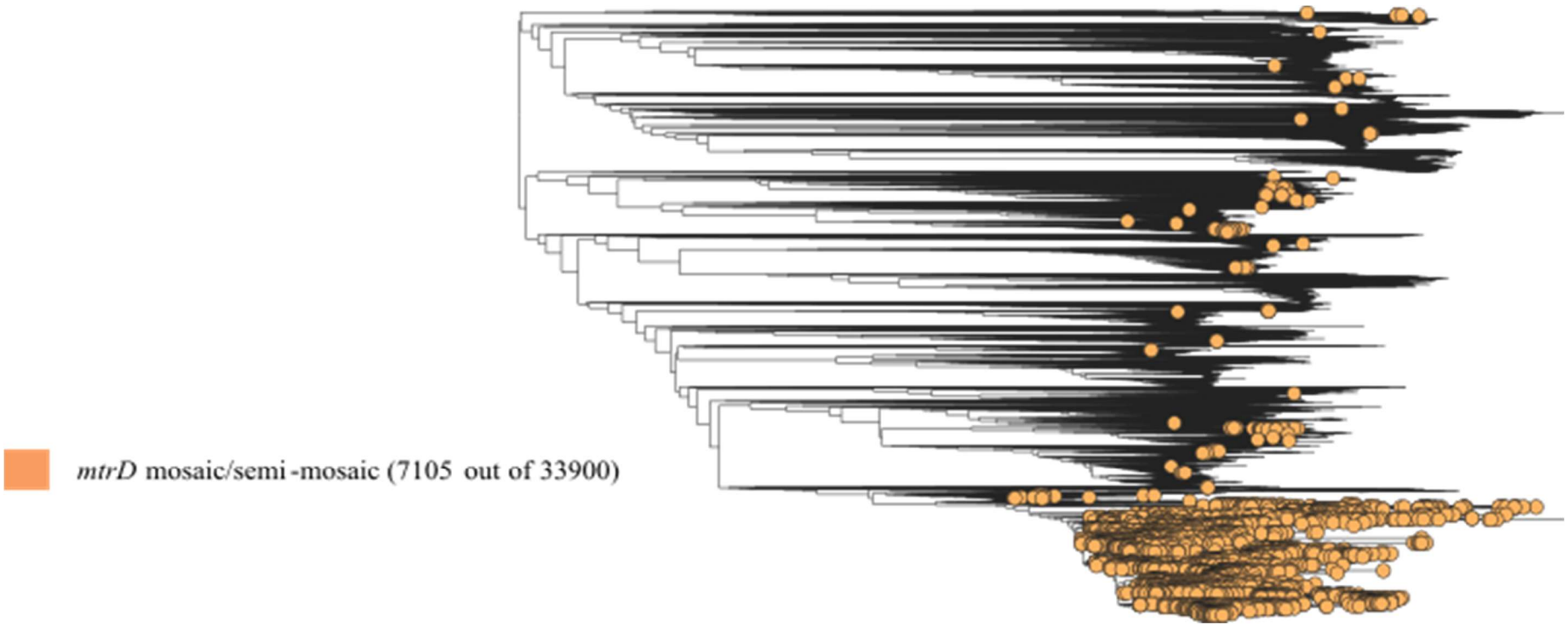
Supplementary Figure 3: **Phylogenetic tree of 51 *mtrD* variants found in the *Neisseria gonorrhoeae* isolates in the EEA, 2020**. For comparison, *mtrD* nucleotide sequences from *N. cinerea* (GenBank: LS483369), *N. flavescens* (GenBank: CP039886), *N. lactamica* (GenBank: CP031253), *N. meningitidis* (GenBank: LR134525), *N. perflava* (GenBank: CP079818), *N. sicca* (GenBank: CP059566), and *N. subflava* (GenBank: CP073119) are shown. The nodes are coloured according to azithromycin MIC_{50} in mg/L. In total, 18, 4, and 29 mosaic, semi-mosaic, and non-mosaic *mtrD* variants were found, respectively. The sequences of all *mtrD* variants and their phylogentic relations as well as annotation can be found at: https://microreact.org/project/mtrd-alleles). EEA=European Economic Area. MIC=minimum inhibitory concentration.



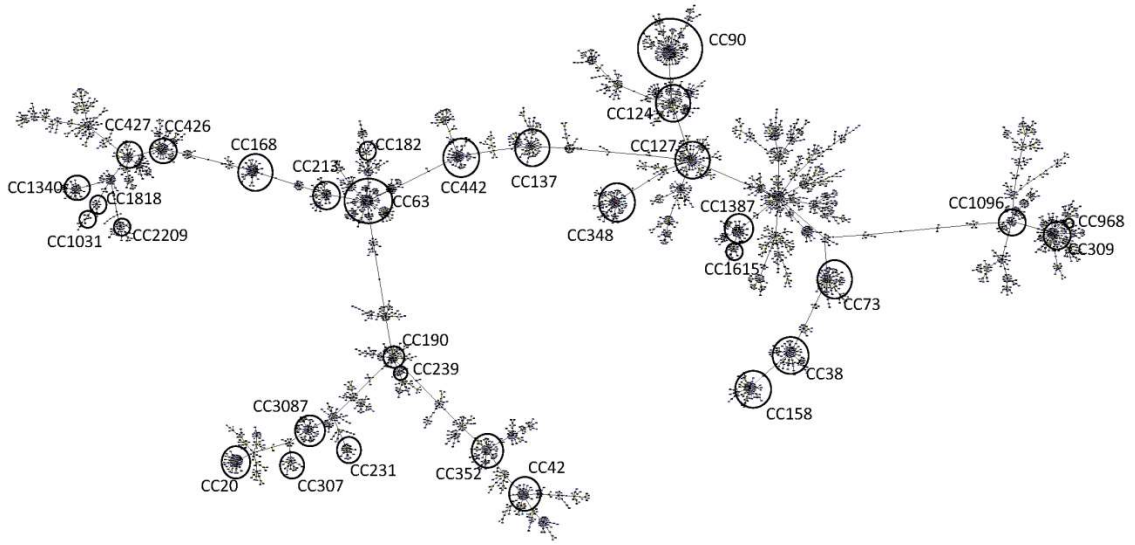
Supplementary Figure 4: **The proportion of *Neisseria gonorrhoeae* NG-STAR CCs in EEA in 2013 (1054 isolates)[4] and 2018 (n=2375)[5] compared to in 2020 (n=1932).** NG-STAR CC=*N. gonorrhoeae* sequence typing antimicrobial resistance clonal complex. EEA=European Economic Area.



Supplementary Figure 5: **Phylogeny of *Neisseria gonorrhoeae* NG-STAR CC1031 isolates in the EEA in 2020 (subtree of the phylogenomic tree in Figure 1).** The size of the circles in the map corresponds to the number of isolates in each country. Azithromycin resistance is shown on right side. NG-STAR CC=*N. gonorrhoeae* sequence typing antimicrobial resistance clonal complex. EEA=European Economic Area.



Supplementary Figure 6: **Core phylogenomics of 33,900 publicly available *Neisseria gonorrhoeae* isolates show that the vast majority of all mosaic/semi-mosaic *mtrD* sequences are in more recently evolved clades of lineage A.**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CC427
CC426
CC168
CC182
CC213
CC1340
CC1818
CC63
CC442
CC137
CC127
CC348
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CC1096
CC968
CC309
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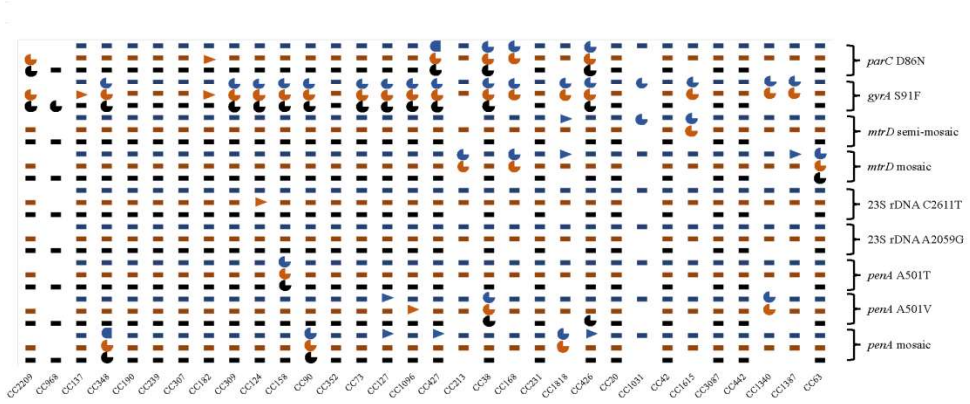
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gyrA S91F
mtrD semi-mosaic
mtrD mosaic
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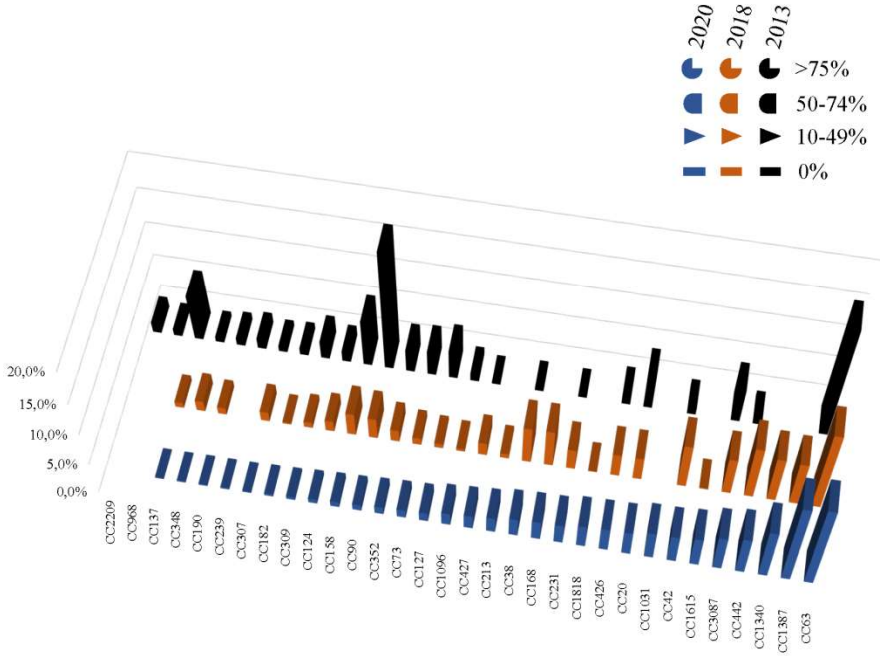
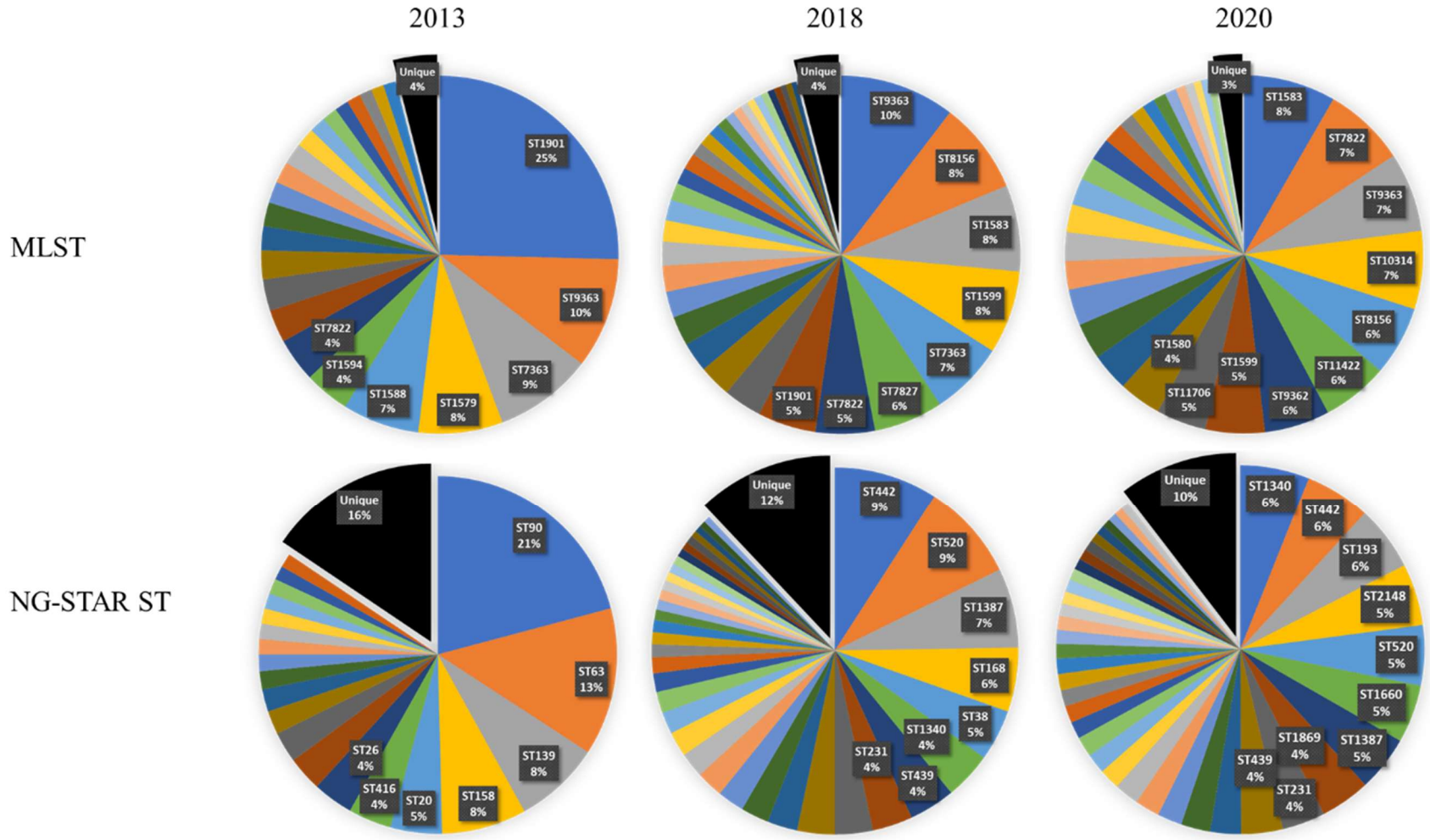
penA A501T
penA A501V
penA mosaic


2020
2018
2013
>75%
50-74%
10-49%
0%
20,0%
15,0%
10,0%
5,0%
0,0%

Supplementary Figure 7: **NG-STAR goeBURST population structure of *Neisseria gonorrhoeae* based on NG-STAR database (downloaded June 26, 2022) with the top NG-STAR CCs from the three Euro-GASP surveys highlighted with circles (figure on top), ordered proportions of the top 20 NG-STAR CCs in EEA in 2013 (1053 isolates),[4] 2018 (n=2374),[5] and 2020 (n=1932) (lower right figure), and proportion of the main AMR determinants for ceftriaxone, cefixime, azithromycin and ciprofloxacin in each NG-STAR CC (lower left figure).** NG-STAR=*N. gonorrhoeae* sequence typing antimicrobial resistance. CC=clonal complex. EEA=European Economic Area. AMR=antimicrobial resistance.



Supplementary Figure 8: **The proportion of *Neisseria gonorrhoeae* MLST and NG-STAR STs found in Euro-GASP genomic surveys in 2013,[4] 2018,[5] and 2020.** The proportion of STs >4% are shown and proportion of unique STs are highlighted in black. MLST=multi-locus sequence typing. NG-STAR=*N. gonorrhoeae* sequence typing antimicrobial resistance. ST=sequence type. Euro-GASP=European Gonococcal Surveillance Programme.

Supplementary Table 1: Distribution of MLST and NG-STAR sequence types, and NG-STAR clonal complexes in the Euro-GASP 2020 genomic survey

Country	Number of isolates	Number of MLST STs (%)*	Most common MLST STs (no. of isolates)	Number of NG-STAR types (%)*	Most common NG-STAR STs (no. of isolates)	Number of NG-STAR CCs (%)*	Most common NG-STAR CCs (no. of isolates)
Austria	69	24 (18·8)	10314 (9), 11422 (9), 1583 (7)	30 (9·4)	1340 (7), 1615 (5), 2885 (5)	21 (26·3)	63 (14), 1387 (10), 1340 (7)
Belgium	149	37 (28·9)	9362 (19), 1583 (16), 11706 (12)	56 (17·5)	1869 (13), 1660 (12), 193 (6)	26 (32·5)	63 (19), 1031 (16), 1340 (16)
Czech Republic	75	17 (13·3)	1599 (30), 8156 (10), 1583 (6)	23 (7·2)	520 (9), 4344 (6), 439 (5)	16 (20·0)	42 (29), 63 (10), 442 (10)
Denmark	108	24 (18·8)	7822 (20), 11177 (19), 1583 (14)	32 (10·0)	568 (22), 1387 (16), 1340 (13)	20 (25·0)	3087 (26), 1387 (19), 1340 (14)
Estonia	2	2 (1·6)	1580 (1), 1583 (1),	2 (0·6)	1340 (1), 3419 (1)	2 (2·5)	1340 (1), 1818 (1)
Finland	76	17 (13·3)	7822 (17), 11370 (11), 11990 (9)	22 (6·9)	2148 (16), 1530 (11), 193 (9)	17 (21·3)	1387 (20), 63 (16), 891 (11)
France	146	32 (25·0)	9362 (15), 11706 (15), 10314 (12)	51 (15·9)	1869 (15), 439 (9), 1387 (10)	32 (40·0)	63 (25), 1387 (22), 1615 (17)
Germany	150	42 (32·8)	9362 (12), 1583 (11), 11984 (10)	65 (20·3)	1660 (12), 563 (9), 193 (8)	35 (43·8)	3087 (18), 73 (12), 1031 (12)
Greece	80	21 (16·4)	11706 (15), 7363 (12), 7827 (8)	31 (9·7)	3736 (13), 2537 (10), 38 (7)	18 (22·5)	1387 (17), 1615 (14), 427 (12)
Hungary	78	21 (16·4)	13489 (15), 11422 (14), 1583 (6)	37 (11·6)	1225 (13), 213 (11), 63 (6)	20 (25·0)	38 (15), 213 (11), 63 (9)
Iceland	39	13 (10·2)	11990 (17), 7822 (3), 10314 (3)	13 (4·1)	725 (18), 436 (3), 2148 (3)	8 (10·0)	20 (18), 1387 (6), 63 (5)
Ireland	99	16 (12·5)	1580 (37), 9363 (15), 7822 (12)	26 (8·1)	1818 (30), 4385 (13), 1387 (10)	12 (15·0)	1818 (30), 63 (23), 1387 (17)
Italy	72	27 (21·1)	10314 (9), 1583 (8), 13956 (8)	41 (12·8)	2724 (8), 1816 (6), 442 (4)	24 (30·0)	1387 (11), 3087 (9), 1340 (7)
The Netherlands	153	31 (24·2)	1583 (21), 10314 (19), 11422 (14)	51 (15·9)	193 (15), 1340 (13), 439 (11)	18 (22·5)	63 (36), 1387 (24), 1340 (23)
Norway	74	22 (17·2)	1588 (13), 8156 (11), 7822 (8)	29 (9·1)	1873 (13), 1387 (8), 4580 (7)	20 (25·0)	1096 (13), 63 (11), 1387 (8)
Poland	20	7 (5·5)	1583 (8), 11422 (5), 9363 (3)	11 (3·4)	1340 (6), 2885 (3), 213 (2)	6 (7·5)	1340 (8), 63 (5), 213 (4)
Portugal	78	31 (24·2)	7822 (8), 11706 (7), 1583 (5)	42 (13·1)	520 (6), 1869 (6), 439 (5)	22 (27·5)	1387 (14), 63 (10), 42 (7)
Slovakia	102	18 (14·1)	1594 (17), 8156 (15), 1901 (10)	36 (11·3)	20 (17), 442 (13), 520 (6)	20 (25·0)	20 (17), 442 (15), 127 (13)
Slovenia	71	13 (10·2)	10314 (24), 9363 (14), 1583 (9)	16 (5·0)	2148 (22), 168 (15), 539 (8)	11 (13·8)	1387 (25), 168 (15), 1340 (9)
Spain	144	35 (27·3)	9363 (15), 8135 (14), 1583 (11)	59 (18·6)	729 (15), 193 (12), 231 (11)	31 (38·8)	63 (21), 3087 (16), 1340 (12)
Sweden	147	33 (25·8)	9362 (33), 7359 (19), 10314 (10)	43 (13·4)	1660 (33), 231 (19), 684 (9)	28 (35·0)	1031 (33), 231 (19), 1387 (12)

Total	1936	128	1583 (145), 7822 (130), 9363 (125)	318	1340 (90), 442 (85), 193 (84)	80	63 (250), 1387 (244), 1340 (148)

MLST=Multi-locus sequence typing. NG-STAR=*Neisseria gonorrhoeae* sequence typing for antimicrobial resistance. Euro-GASP=European Gonococcal Antimicrobial Surveillance Programme. ST=Sequence type. CC=clonal complex.

*Proportion of MLST STs, NG-STAR STs, and NG-STAR CCs found per country out of the total number of STs or CCs found in the whole collection.

Supplementary Table 2: **Number of mosaic, semi-mosaic and non-mosaic *mtrD* variants in the Euro-GASP 2020 genomic survey**

mtrD variant	Non-mosaic	Mosaic	Semi-mosaic	Total
***mtrD* mosaic 2**		201		201
Variant C		112		112
Variant 34		29		29
Variant 35		19		19
Variant 32		6		6
Variant 52		6		6
Variant 49		3		3
Variant 50		3		3
Variant 7		2		2
Variant 33		2		2
Variant 53		2		2
Variant 30		1		1
Variant 8		1		1
Variant 31		1		1
Variant 36		1		1
Variant 46		1		1
Variant 48		1		1
Variant 54		1		1
Variant 13			104	104
Variant 39			84	84
Variant 40			1	1
Variant 41			1	1
Variant 10	366			366
WHO N variant	191			191
Variant 59	164			164
Variant 6	141			141
WHO M variant	138			138
Variant 17	57			57
Variant 21	49			49
Variant R	45			45
Variant 62	34			34
Variant 44	28			28
Variant T	28			28
WHO U variant	26			26
WHO O variant	17			17
Variant 37	15			15
Variant 56	11			11
Variant 58	8			8
Variant 38	6			6
Variant 42	5			5
Variant 60	5			5
Variant 24	3			3
Variant 47	3			3
Variant 45	2			2
Variant 57	2			2
Variant 29	1			1
Variant 43	1			1
Variant 51	1			1
Variant 55	1			1
Variant 61	1			1
Variant Z	1			1

***Supplementary Table 3*: Segregating sites, nucleotide diversity, and Tajima's Neutrality Test (D)[22] in 2020 compared to 2018, and the average COVID-19 stringency index in 2020 in 19 EEA countries**

Country[a]	Year	Segregating sites	Nucleotide diversity	Tajima's D	Average COVID-19 stringency index in 2020 (https://ourworldindata.org/covid-stringency-index)
Austria	2018	25,401	0·0026	0·406	55·4
	2020	18,774	0·0022	-0·320	
Belgium	2018	25,567	0·0026	-0·174	56·3
	2020	16,092	0·0008	-1·549	
Czech Republic	2018	21,168	0·0025	0·507	51·7
	2020	15,331	0·0017	-0·019	
Denmark	2018	22,394	0·0028	0·711	50·0
	2020	21,138	0·0025	0·049	
Finland	2018	22,067	0·0024	-0·245	42·0
	2020	18,365	0·0021	0·274	
France	2018	20,651	0·0026	-0·024	57·9
	2020	19,703	0·0019	-0·348	
Germany	2018	25,660	0·0025	-0·083	55·7
	2020	30,241	0·0026	-0·408	
Greece	2018	21,213	0·0024	0·228	64·3
	2020	20,686	0·0020	-0·376	
Hungary	2018	23,415	0·0027	0·290	58·3
	2020	20,350	0·0022	0·060	
Iceland	2018	18,686	0·0025	0·376	45·0
	2020	13,705	0·0022	1·150	
Ireland	2018	28,423	0·0023	0·050	67·0
	2020	15,315	0·0016	-0·375	
Italy	2018	23,664	0·0024	0·017	70·4
	2020	17,899	0·0019	-0·384	
The Netherlands	2018	30,274	0·0027	-0·099	59·2
	2020	17,731	0·0015	-0·454	
Norway	2018	25,572	0·0026	0·029	48·2
	2020	21,389	0·0026	0·366	
Portugal	2018	24,605	0·0027	0·157	64·3
	2020	22,107	0·0024	0·049	
Slovakia	2018	20,860	0·0025	0·423	54·9
	2020	8,470	0·0005	-1·426	
Slovenia	2018	21,555	0·0023	0·177	54·8
	2020	9,847	0·0010	0·337	
Spain	2018	29,202	0·0026	-0·189	61·4
	2020	25,793	0·0022	-0·487	
Sweden	2018	34,370	0·0026	-0·029	53·5
	2020	22,632	0·0022	-0·580	

EEA=European Economic Area

[a]Poland (n=20) and Estonia (n=2) were excluded from the analysis due to few isolates.

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