

Genomic variation landscape of the human gut microbiome

Siegfried Schloissnig^{1*}, Manimozhayan Arumugam^{1*}, Shinichi Sunagawa^{1*}, Makedonka Mitreva², Julien Tap¹, Ana Zhu¹, Alison Waller¹, Daniel R. Mende¹, Jens Roat Kultima¹, John Martin², Karthik Kota², Shamil R. Sunyaev³, George M. Weinstock² & Peer Bork^{1,4}

Whereas large-scale efforts have rapidly advanced the understanding and practical impact of human genomic variation, the practical impact of variation is largely unexplored in the human microbiome. We therefore developed a framework for metagenomic variation analysis and applied it to 252 faecal metagenomes of 207 individuals from Europe and North America. Using 7.4 billion reads aligned to 101 reference species, we detected 10.3 million single nucleotide polymorphisms (SNPs), 107,991 short insertions/deletions, and 1,051 structural variants. The average ratio of non-synonymous to synonymous polymorphism rates of 0.11 was more variable between gut microbial species than across human hosts. Subjects sampled at varying time intervals exhibited individuality and temporal stability of SNP variation patterns, despite considerable composition changes of their gut microbiota. This indicates that individual-specific strains are not easily replaced and that an individual might have a unique metagenomic genotype, which may be exploitable for personalized diet or drug intake.

With the increasing availability of individual human genomes, various theoretical and practical aspects of genomic variation can be deduced for individuals and the human population as a whole^{1,2}. Like sequenced human genomes, the number of human gut metagenomes (currently mostly derived from Illumina shotgun sequencing of stool samples) is increasing exponentially. Given the importance of the gut microbiota in human health^{3,4} and a growing number of studies reporting associations between gut microbiota and diseases^{5–8}, an understanding of genomic variation in gut microbial populations will probably trigger applications towards human well-being and disease.

For example, in the common gut commensal bacterium *Escherichia coli*, just three point mutations in two genes can confer clinically relevant antibiotic resistance⁵, and natural variation in a single gene can lead to pathogenic adaptation⁸. Even within pathogenic species in the gut, closely related coexisting strains can exhibit different pathogenic potentials due to minor genomic variation⁷. These examples illustrate how genomic variation within gut microbes could confer phenotypes that require personalized care or treatment of the host.

Studies based on 16S ribosomal RNA gene surveys or whole metagenome shotgun sequencing characterized taxonomic and functional compositions of healthy individuals' and intestinal bowel disease patients' gut microbiota at the genus or species level^{6,9–12}. Variation in taxonomic abundance as well as functions encoded by these gut microbiota have been described between individuals^{6,11} and used to stratify individuals according to their gut community compositions into enterotypes¹³. However, genomic variation within species, which leads to their phenotypic diversity and adaptations to different environments, has only been studied in a few taxa, such as *Citrobacter* spp⁷.

An early landmark study on a small data set described metagenomic variation in an acidic biofilm microbiome of low complexity¹⁴. The population structure for one species in that habitat was studied and positive selection was observed in some genes¹⁵. Another recent study resolved multiple clinical isolates of methicillin-resistant

Staphylococcus aureus and delineated its epidemiology and microevolution based on genomic variation¹⁶. With the availability of hundreds of deeply sequenced human gut metagenomes^{9,11,17}, sufficient data are becoming available for quantitative analyses of the genetic structure of complex microbial communities, allowing the study of many species at the same time.

Here we analysed 1.56 terabases of sequence data from 252 stool samples from 207 individuals (Supplementary Table 1 and Supplementary Notes) obtained from the MetaHIT project (71 Danish, 39 Spanish; all sampled once¹¹), the NIH Human Microbiome Project (94 US samples; 51 individuals sampled once, 41 sampled twice and 2 sampled three times⁹), and Washington University (three US samples; all sampled once¹²). Our goals were to: (1) develop a framework for genomic variation analysis using metagenomic shotgun data; (2) gather basic knowledge on the genomic variation landscape in gut metagenomes; and (3) gain insights into the individuality, temporal stability and biogeography of metagenomic variation.

Framework for metagenomic variation analysis

We used 1,497 prokaryotic genomes to generate a set of reference genomes (Supplementary Table 2) for the analysis of genomic variation in gut microbial species in 252 samples (on average 6.2 ± 4.1 gigabases (Gb) were analysed). Pairwise comparisons of 40 universal marker genes^{18,19} identified in these genomes were performed to create a set of 929 clusters based on a 95% DNA identity threshold recommended for identifying species²⁰. The genome recruiting the highest number of reads in a cluster was selected as reference for that species (see Methods and Supplementary Information).

Using the same 95% identity threshold, we mapped 7.4 billion metagenomic reads (42% of the total, 91% thereof uniquely) with an average length of 80 base pairs (bp) to the 929 reference genomes (Supplementary Tables 1 and 3). To avoid mapping artefacts (for example, caused by high coverage of prophages), we required $\geq 40\%$

¹European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany. ²The Genome Institute, Washington University School of Medicine, 4444 Forest Park Avenue, St Louis, Missouri 63108, USA. ³Division of Genetics, Department of Medicine, Brigham & Women's Hospital & Harvard Medical School, Boston, Massachusetts 02115, USA. ⁴Max Delbrück Centre for Molecular Medicine, D-13092 Berlin, Germany.

*These authors contributed equally to this work.

of each reference genome to be covered by reads (corresponding to the gene content similarity between two strains of *E. coli*²¹). The resulting 101 prevalent species with base-pair coverage from $12\times$ to $32,400\times$ (Fig. 1 and Supplementary Fig. 1) were subjected to genomic variation analysis.

To enable comparative analyses in multiple metagenomes and to identify low-frequency variants not detected when analysing them individually, we used multi-sample calling²² to identify SNPs, short insertions/deletions (indels; 1–50 bp) and structural variations (>50 bp) in each genome, although structural variations were largely underestimated due to small insert sizes (Supplementary Information). We only called variants with allele frequency $\geq 1\%$ (the conventional definition of polymorphism²) and supported by ≥ 4 reads. False-positive rates were estimated at 0.71% for SNPs and 1.04% for structural variations (Supplementary Information, Supplementary Tables 4 and 5, and Supplementary Fig. 2).

Genomic variation in prevalent gut species

We identified 10.3 million SNPs in 101 genomes (3.1% of the total 329 Mb) across 252 samples from 207 subjects, almost as many as the 14.4 million SNPs recently identified in 179 human genomes². Within an individual the rate was lower (on average 1.21%, see Supplementary Table 6), yet SNPs kb^{-1} increased with base-pair coverage when samples were pooled (Supplementary Fig. 3). We also identified 107,991 indels and 1,051 structural variants in these 101 species (Supplementary Information). Their relative ratios to SNPs (10,485 short indels and 102 structural variants per million SNPs) were robust across species and individuals (Supplementary Fig. 4). Subsequent

analyses were restricted to SNPs due to their orders of magnitude higher count over other variation types.

We annotated the genes of the prevalent genomes using orthologous groups from eggNOG²³ (Supplementary Information) and found that the orthologous groups with the highest SNP density were enriched in functions related to conjugal transfer of antibiotic resistance (Supplementary Table 7). For example, the orthologous group with the highest average SNP density across samples was the clindamycin resistance transfer factor *btgA* (NOG119724), required for conjugative transmission of plasmids. Mutations commonly accrued from the process of conjugation may account for increased diversity among conjugation-associated functions²⁴. Additionally, CRISPR-associated proteins, responsible for conferring resistance in bacteria, were also found among the orthologous groups with high SNP densities (Supplementary Information and Supplementary Table 7).

The large number of SNPs provided the opportunity to compare, for the first time at such scale, the evolution of different coexisting species across a large cohort of individuals. To evaluate selective constraints in these species in their natural habitat, we estimated the ratio of non-synonymous to synonymous polymorphism rates^{25,26} (pN/pS) within each species in every sample (Fig. 1 and Supplementary Information). pN/pS characterizes selective constraint at the level of a population contrary to the more commonly used dN/dS that characterizes it between individual species²⁶. To validate pN/pS ratios, we estimated genetic variation using the sample-size-independent nucleotide diversity π , and found that π is highly correlated with SNPs kb^{-1} (Fig. 1 and Supplementary Fig. 5). The derived measures of $\pi(N)/\pi(S)$ and $\pi(\text{non-degenerate sites})/\pi(\text{fourfold degenerate sites})$, the latter of

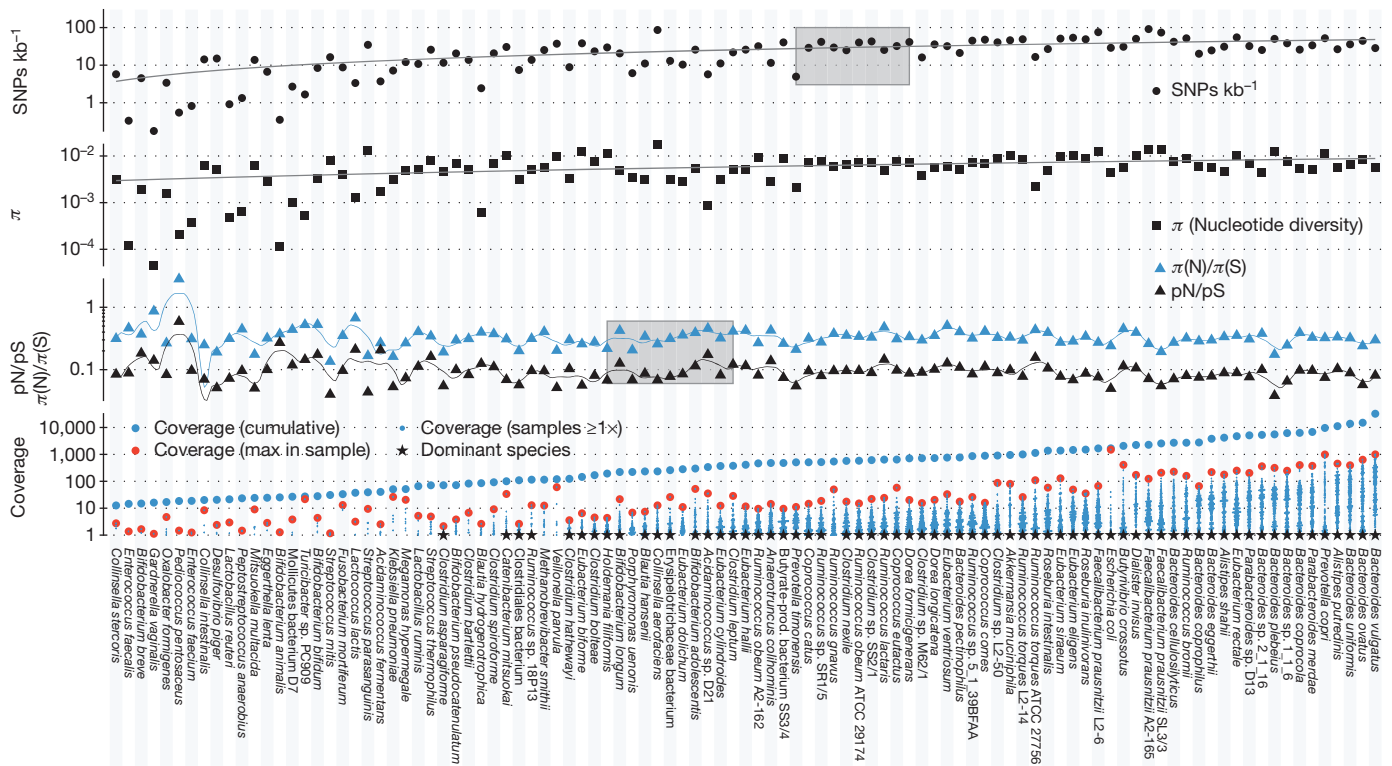


Figure 1 | Genomic variation statistics for 101 gut microbial species prevalent in 252 samples from 207 individuals. Genomic variation statistics were calculated for 101 prevalent gut microbial species, operationally defined as having $\geq 10\times$ cumulative (over all samples) base-pair coverage with at least one sample exhibiting a genome coverage of $\geq 40\%$. The 66 dominant species (indicated by an asterisk), which account for 99% of the mapped reads, were used for analyses that required high base-pair coverage. Species names are given without strain specifications unless this would result in duplicate entries. The blue point cloud plots show the coverages ($\geq 1\times$) in all samples, with the blue dot above indicating the cumulative coverage and the red dot the maximum

coverage across all samples. Grey shaded areas indicate the level of base-pair coverage at which abundance effects have only minor effects on SNPs kb^{-1} and pN/pS ratios of the pooled samples (Supplementary Information). SNP counts appear to saturate at approximately $500\times$, with minor increases at higher coverages probably due to the sampling of rare variants at low rates. In individual samples, pN/pS is largely stable from a coverage of $10\times$ onward (Supplementary Fig. 7), corresponding to approximately $200\times$ cumulative coverage in our sample set. Nucleotide diversity π follows SNPs kb^{-1} closely, as does the derived measure of $\pi(N)/\pi(S)$ with respect to pN/pS.

which is less dependent on specific properties of mutation spectra such as transition and transversion ratios, were coherent with pN/pS (Supplementary Fig. 6).

The pN/pS ratio of a genome within a sample remained stable at coverages higher than $10\times$ (Supplementary Fig. 7)—yet another indication of few false SNP calls—and was on average 0.11, but varied considerably for different species (0.04 to 0.58) in accordance with dN/dS ratios estimated independently in a number of interspecies comparisons between closely related bacteria and archaea^{27,28}.

pN/pS across gut species and individuals

Because meaningful comparison of genomic variation requires both breadth (across samples) and depth (in number of base pairs) of sequencing, we focused on the 66 most dominant species that attracted $>99\%$ of the reads (Fig. 1). Their relatively low pN/pS ratios were constant across different hosts (Fig. 2a and Supplementary Table 8), which may indicate similar selective constraints across individuals. Thus, the evolution of gut species is probably dominated by long-term purifying selection and drift rather than rapid adaptations to specific host environments. The wide range of these ratios across species may suggest that different gut species face different evolutionary constraints.

To investigate how different gut species respond to the pressure from the gut environment, we compared the pN/pS ratios of individual genes in *Roseburia intestinalis* and *Eubacterium eligens*, which differed considerably in their overall mean pN/pS ratios (0.236 (*R. intestinalis*) versus 0.131 (*E. eligens*) from 106 and 147 samples, respectively) despite having comparable average base-pair coverages (Supplementary Information). Whereas 75% of the genes in *R. intestinalis* had systematically higher pN/pS ratios compared to their orthologues in *E. eligens*, few others showed considerable deviations (Fig. 2b and Supplementary Table 9), indicative of differing evolutionary constraints for these genes. For example, *galK*, the gene encoding galactokinase, an essential enzyme in the Leloir pathway for galactose metabolism in most organisms²⁹, was among the lowest in terms of pN/pS ratio in *R. intestinalis* but among the highest in *E. eligens* (0.03 and 0.48, respectively; Fig. 2b, c). Although present in *E. eligens*, this gene may not exert its main function (see also ref. 30), as *E. eligens* cannot ferment galactose, nor the galactose-containing disaccharides lactose and melibiose³¹. On the other hand, *R. intestinalis* is known to ferment melibiose³², indicating that its *galK* gene is functional (Supplementary Information). Thus, the same gene can be under tight negative selection in one species but under more relaxed negative selection in another.

Our framework allowed us additionally to obtain information on all genes in each sample (Supplementary Information). As expected, we found that housekeeping genes had usually lower pN/pS ratios. For example, the DNA-dependent RNA polymerase β -subunit gene was consistently among the genes exhibiting the lowest pN/pS ratios across samples and species (Supplementary Table 10). Less obvious examples included genes related to type IV secretion systems used to transfer DNA between microbes³³ and involved in host interactions of both pathogenic³³ and commensal bacteria³⁴, specifically in anti-inflammatory responses and immune modulation³⁵. The low pN/pS ratio of genes related to type IV secretion systems suggests that maintaining genome plasticity and antibiotic resistance through conjugative transposition is essential in the constantly changing environment of the gut and that the interaction with the host immune system is under purifying selection (Supplementary Table 10). We also found a few conserved unknown, but apparently gut-microbe-specific, proteins that exhibited low pN/pS ratios, suggesting that they perform important yet hitherto unexplored functions (Supplementary Table 11).

Among the genes or orthologous groups with consistently the highest pN/pS ratios were many transposases and antimicrobial resistance genes including the gut-specific gene bile salt hydrolase (BSH)³⁶ (Supplementary Table 10). Conjugated bile acids (CBAs) secreted

by the hosts repress microbial growth and upregulate the host mucosal defence system. BSHs are involved in the initial reaction in the metabolism of CBAs by gut microbes³⁶. Their high pN/pS ratio may be indicative of the genomic plasticity necessary to metabolize and survive the variety of different bile acids present in the gut³⁷.

Temporal stability of individual SNP patterns

Several studies on adult human gut microbial samples from a few individuals have suggested that within-individual differences over time are smaller compared to between-individual differences in microbial species composition and abundance^{38–40}. Within a larger cohort, individuality of host-associated microbiota has been reported on the basis of 16S rRNA gene profiling of fingertip-associated communities⁴¹, whereas other studies on a few samples have investigated the persistence of specific strains over time^{42,43}. However, intra-species variation at nucleotide resolution at the whole-genome level and accompanying changes in species abundances within the human gut over long time periods (>1 year) have not been studied yet in large cohorts. It is unclear if the concept of resident strains is common to other prevalent species, if host-specific strains are retained over time, and how fast they evolve inside the gut environment.

To explore these questions, we used 88 gut metagenomes from 43 healthy US subjects (a subset of our cohort) from whom at least two samples were obtained at different time points with no antibiotics treatment in between (Supplementary Table 12). To measure how similar the subpopulations (strains) of the dominant species were between two samples, we estimated the fixation indices (F_{ST}) between the populations (Supplementary Information). Because this measure depends on allele frequencies, which cannot be determined accurately at low base-pair coverage, we also estimated the fraction of alleles shared between the samples out of all polymorphic sites (only 49 genomes that accrued 40% genome coverage in at least two samples were used and genomes with $>10\times$ base-pair coverage were down-sampled to $10\times$; Supplementary Information and Supplementary Fig. 3). Because the fraction of shared SNPs depends on the number of variable sites, we developed a heuristic allele sharing similarity score that takes into account both the number of variable sites and the fraction of shared alleles (Supplementary Information).

When we compared all 252 samples, F_{ST} was significantly lower and allele sharing significantly stronger between different samples from the same individuals than between samples from different individuals (Mann–Whitney U -test: $P < 0.001$ for both; see Fig. 3a, b and Supplementary Information). The same trend was observed, albeit much weaker, based on species compositions (Fig. 3c), in line with previous observations from microbial composition-based results^{38–40}. Intra-individual variation being smaller than inter-individual variation does not require that samples from the same individual are more similar to each other than to any other sample in the tested cohort. Our results showed for both measures of variation similarity that all but one of the 88 multi-time-point samples had the highest similarity to another sample from the same individual, which was not true when comparing species abundance over time (Fig. 3c and Supplementary Information). This indicates that species abundance in gut microbiota cannot serve as a fingerprint of an individual whereas variation patterns might.

We also tested whether differences in F_{ST} and allele sharing decreased over time, which may indicate a divergence of the strains or a horizontal transmission of strains from the environment; however, the individual-specific variation patterns remained stable over all of the time intervals monitored (Fig. 3). Although this stability should be verified for longer periods as well as when antibiotic treatment or other gut microbiota-challenging events have taken place, our observation indicates that healthy human individuals retain specific strains (see also Supplementary Tables 12 and 13) for at least 1 year.

In contrast to the strong evidence for individuality and temporal stability of SNP patterns, we did not observe a significant geographical

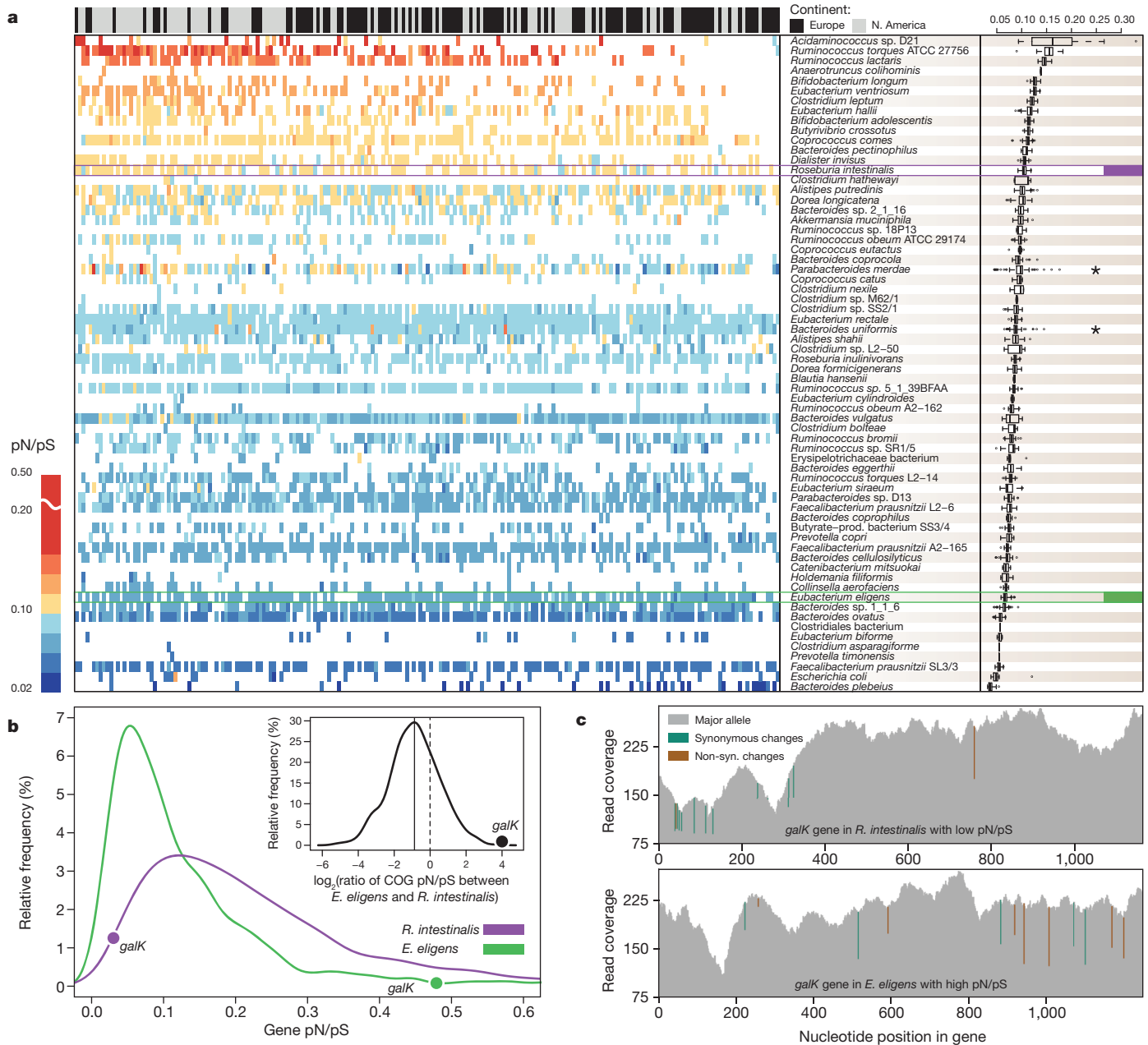


Figure 2 | pN/pS ratios of 66 dominant species reveal more variation between species than between individuals. **a**, A heat map of pN/pS ratios for the 66 dominant species (rows) and 207 individuals (columns; only the first time-point per individual) is shown and summarized by species (box plots on the right). Rows and columns are ordered by their mean pN/pS ratios, which vary considerably between species, but have a tighter bandwidth across samples. Two genomes that are exceptions to this trend (indicated by an asterisk) might indicate higher strain diversity. The panel above the heat map indicates the continent of residence for each individual. A significant difference was found in the mean pN/pS ratios between the two continents, although this is probably an effect of lower sequencing depths of European samples (Supplementary Table 8) that leads to missing data points in some samples (see,

separation between European and US samples (Supplementary Fig. 8 and Supplementary Table 14). This indicates that long-term horizontal transmission of at least some dominant gut microbial strains cause geographical mixing over time. The strongest continental separation, based on F_{ST} , was seen in *Bacteroides coprocola* (Fig. 4), which was also the only genome with sufficient amounts of data that showed continental separation based on the allele sharing score (Supplementary Fig. 8 and Supplementary Tables 14 and 15).

for example, top-right corner). **b**, The distributions of average pN/pS ratios of individual genes from *Roseburia intestinalis* and *Eubacterium eligens* (both highlighted in **a**) illustrate that, although base-pair coverages are similar, the pN/pS ratio of *R. intestinalis* is higher in general. The relative pN/pS ratios of orthologous groups in the two species are shown in the inset, with the average \log_2 ratio indicated by the solid line and the random expectation by the dashed line. Outliers can be revealed this way, like the galactokinase gene (*galK*), the pN/pS of which is among the lowest in *R. intestinalis* and the highest in *E. eligens*. **c**, Illustration of low and high pN/pS ratios in *galK* genes from *R. intestinalis* (top panel) and *E. eligens* (bottom panel). The cumulative read coverage is shown in grey with synonymous (green) and non-synonymous (brown) changes marked at the nucleotide positions they occur.

Discussion

We have established a framework for gut microbial genomic variation analysis using metagenomic data and identified in a single analysis—involving 252 stool samples from 207 human individuals—almost as many SNPs in the human gut microbiome as the 1000 Genomes Project recorded in 179 human individuals over several years². The stable pN/pS ratios of gut microbial species across individuals suggest that host conditions (such as diet, genetic differences, and immune

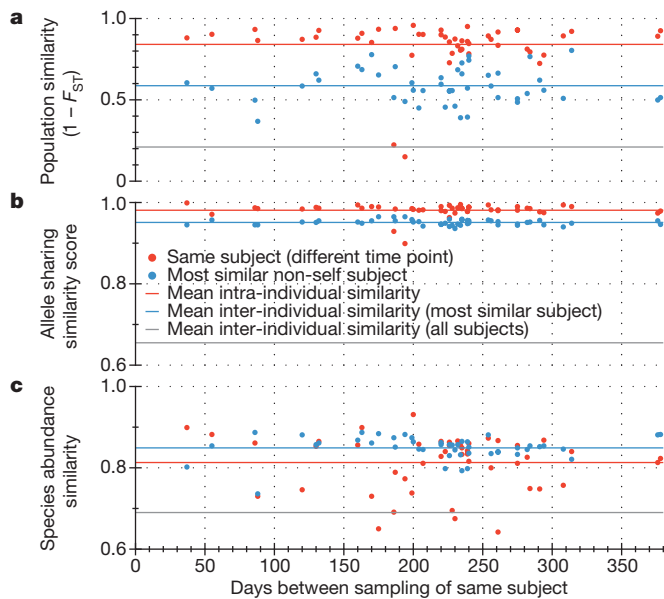


Figure 3 | Individuality and temporal stability of genomic variation patterns. a–c, Samples from 43 individuals that were sampled at different time intervals (red dots) were compared with the most similar sample from a different individual (blue dots) in terms of population similarity that takes allele frequencies into account (a), allele sharing similarity score that takes SNP counts and the ratio of shared SNPs into account (b) (Supplementary Information) and species abundance similarity measured using the Jensen–Shannon distance¹³ (c). The most similar sample is the one with the lowest F_{ST} value in a, the highest allele sharing similarity score in b and the lowest Jensen–Shannon distance in c. The three similarity measures are plotted against the number of days between the sampling time points. The mean across all intra-individual, best inter-individual and all inter-individual similarities is shown as red, blue and grey lines, respectively. For both population similarity and allele sharing similarity between samples from the same individual, all but one sample (resulting in two outliers due to comparisons with two other time-points, see Supplementary Table 12) shared the highest similarity with another sample of the same individual, providing strong evidence for individuality of SNP sharing patterns. No decline of similarity over time could be observed.

tolerance) have a minor influence on the evolution of species compared to constraints common to the human population (such as gut physiology, anaerobic conditions and pH). In the 66 dominant species, the analysis of more than 229,000 genes comprising about 8,000 orthologous groups pinpointed consistently fast or slow evolving genes across individuals (Supplementary Table 10). However, further studies are needed to interpret different selection types at the gene level.

The availability of time-point data revealed that individual-specific variation patterns were remarkably stable over time (Fig. 3a, b), which

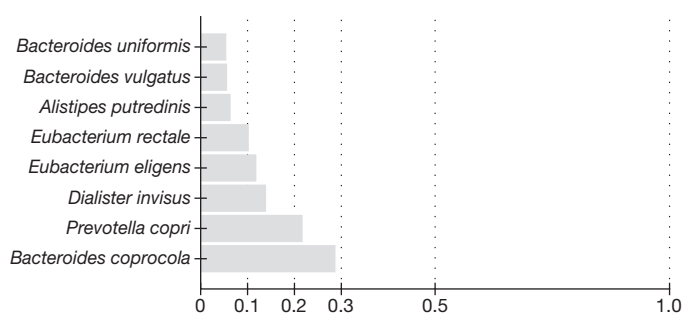


Figure 4 | Inter-continental comparison of gut microbial species. Between-continent F_{ST} values for eight genomes with ≥ 10 samples representing each continent are shown. *Bacteroides coprocola* was the species with the highest F_{ST} value, implying a separation between the *B. coprocola* populations in Europe and North America (see also Supplementary Information; all data are available in Supplementary Table 14).

was much less the case for similarities in species abundance—for almost 60% of the samples, a sample from a different individual was the most similar (Fig. 3c). Thus, the metagenomic variation patterns observed here support the hypothesis that a healthy individual retains specific strains for extended periods of time. This suggests that each individual has a metagenomic variation profile that could be unique even in very large cohorts. It should be noted that the maximal sampling period was only 1 year, and 43 individuals might not be sufficient to trace horizontal transmissions of strains. The likelihood of the latter is supported by the apparent absence of clear continental stratification (despite different sampling and sequencing protocols of the European and the US samples), although for one out of eight species analysed, *Bacteroides coprocola*, we provide preliminary evidence (Fig. 4 and Supplementary Fig. 8). Geographical stratification has been described for *Helicobacter pylori*^{44,45}, and weak but detectable signals have also been observed in some bacterial pathogens^{46,47}. Thus, we expect more gut microbial stratification patterns to emerge when larger data sets under standardized sampling and sequencing protocols become available, although it remains to be tested which factors (such as geographical separation, diseases, host-genetic and life-style/diet factors) shape the distribution of gut microbial strains and segregating SNPs within the population. The absence of clear geographical stratification implies that stable differences in variation patterns of gut species are not explained by large-scale structures of local microbial populations. They may rather be a result of genetic drift due to population bottlenecks that could occur not only during the colonization of the infant gut but also by processes causing community shifts during adult life stages, followed by a rapid population growth accompanied by purifying selection. This model suggests that the source of genetic variation in human gut microbial populations is less likely to be new mutations within the host than the variation in the initial colonizing populations or transmissions from the environment. This would imply that most allelic variants analysed in this study segregate at timescales greatly exceeding human generation time.

The introduction of large-scale variation analysis in metagenomic data of complex communities and the discovery of individual metagenomic variation profiles open up several applications. It is now possible to screen *in silico* for many pathogenic or antibiotics resistance variants in the population. Once a sample has been analysed, the data can also be used in the future given the temporal stability of SNP profiles. As it took years to identify marker genes and variations for diseases or phenotypes in the human genome, the variation landscape uncovered here can only be seen as the beginning to find molecular biomarkers including particular variants that reveal useful information for human health and well-being.

METHODS SUMMARY

Mapping to non-redundant genomes. A reference genome set representing 929 species was derived from a total of 1,497 published prokaryotic genomes, based on a median sequence identity of 95% in 40 universal, single-copy marker genes^{18,19}. Metagenomic reads from 252 samples were aligned to these 929 genomes using the same 95% sequence identity cutoff.

Coverage. For each genome, we calculated the sample-specific base-pair coverage and the number of bases of the genome covered by at least one read. For a genome to be considered we required a cumulative depth of coverage of $\geq 10\times$ across all samples. To remove species that are not present in our cohort, yet attract reads due to highly conserved regions, we required at least 40% breadth of the genome coverage (the criterion for the species to be considered present) from at least one sample.

Variation detection. We performed SNP calling on the pooled samples and only considered bases with a quality score ≥ 15 . We required SNPs to be supported by ≥ 4 reads and to occur with a frequency of $\geq 1\%$. Structural variations were detected using Pindel⁴⁸. False-positive rates in SNP and structural variation detection were estimated using nonsense and frameshift mutations in 40 essential single copy marker genes.

π and F_{ST} . We estimated nucleotide diversity (π) and fixation indices (F_{ST}) on the basis of allele frequencies.

pN/pS ratio. SNPs occurring in coding regions were classified as synonymous or non-synonymous. Genes from the non-redundant genomes were annotated using eggNOG orthologous groups allowing calculation of pN/pS ratios at the level of genomes, orthologous groups and genes.

Pairwise sample comparisons. Similarity in strain populations between two samples was estimated using a similarity score based on shared SNPs and using F_{ST} .

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Supplementary Information is available in the online version of the paper.

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Author Information Single nucleotide polymorphism data have been submitted to dbSNP under accession numbers ss539238913–ss549853572. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.B. (bork@embl.de) or G.M.W. (gweistn@genome.wustl.edu).