Resequencing of 200 human exomes identifies an excess of low-frequency non-synonymous coding variants

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Targeted capture combined with massively parallel exome sequencing is a promising approach to identify genetic variants implicated in human traits. We report exome sequencing of 200 individuals from Denmark with targeted capture of 18,654 coding genes and sequence coverage of each individual exome at an average depth of 12-fold. On average, about 95% of the target regions were covered by at least one read. We identified 121,870 SNPs in the sample population, including 53,081 coding SNPs (cSNPs). Using a statistical method for SNP calling and an estimation of allelic frequencies based on our population data, we derived the allele frequency spectrum of cSNPs with a minor allele frequency greater than 0.02. We identified a 1.8-fold excess of deleterious, non-synonymous cSNPs over synonymous cSNPs in the low-frequency range (minor allele frequencies between 2% and 5%). This excess was more pronounced for X-linked SNPs, suggesting that deleterious substitutions are primarily recessive.

Next-generation technologies have reduced the costs of high-throughput sequencing by several orders of magnitude and have allowed for the whole-genome sequencing of several human genomes. However, whole-genome sequencing of the large numbers of individuals needed for studies of population genetics or trait associations remains unaffordable. One alternate approach, taken by the 1000 Genomes Project, is low-pass sequencing (with an average of fourfold genome sequencing depth per sample) of the whole genomes of a large number of individuals. This approach may be useful in identifying population genetics patterns by combining data from the whole sample population.

Exome sequencing through use of targeted sequencing based on array capture is another alternative approach that allows researchers to concentrate their sequencing efforts on the complete set of coding exons of the human genome; using this approach, researchers are perhaps more likely to include functionally important regions. Recent studies have successfully applied targeted capture and exome sequencing to identify genetic changes involved in Mendelian diseases. Such exome-capture sequencing data can now be generated from large population samples. This provides unprecedented opportunities to both characterize the impact of natural selection and to better understand the role of low-frequency variants in the pathogenesis of human diseases. Here we used modest-depth sequencing of the exomes of 200 individuals of European ancestry from Denmark with an average of 12-fold sequencing depth (defined as total number of uniquely mapped bases divided by the full length of target region, which was approximately 34 Mb) per sample to discover new, low-frequency variants by aggregating data from all 200 individuals. With this intermediate design between low-pass population sequencing and deep individual sequencing, we aimed to derive a high-resolution allele frequency spectrum of cSNPs with a minimum allele frequency of 0.02 to characterize the distribution of allele frequencies in a human population and to use this distribution to make inferences about the effect of natural selection in the human genome.

We used the NimbleGen 2.1M Exon Capture Array to capture 34 Mb of the human genome, which included the coding sequences of 18,654 variants.
(92.8%) well-annotated genes together with stretches of untranslated or intronic flanking sequences (Online Methods and Supplementary Note). Targeted regions primarily contained exonic sequences, which we collectively refer to here as an exome. We sequenced DNA from 200 individuals from Denmark with an average coverage of ×12 to ×18 for each individual exome (Supplementary Table 1). A total of 95.8 Gb of high-quality data aligned to the targeted regions with a per-base mismatch rate of 1% (Table 1) and covered the human exome with an average depth of approximately 2,800-fold. For each sample individual, on average, about 95% of the target regions were covered by at least one read and more than 60% of the target regions were covered by at least ten reads, which is in agreement with previous studies. Only 8.8% of the total 34-Mb target regions were covered by less than 600 reads due to systematic bias in the hybrid capturing experiment. Going forward, we only used the regions passing this 600-fold read-depth criterion for estimation of allele frequencies. In addition, we only used SNPs with estimated minor allele frequency (MAF) > 0.02.

As the sequencing depth was not adequate to call genotypes of each individual accurately, we developed a SNP calling and frequency estimation method based on population data that simultaneously used genotype likelihood information from all individuals. This approach significantly increased the statistical power to detect SNPs and frequency estimations (Supplementary Note). Applying this method, we detected 121,870 high-quality SNPs with a false positive rate of 2% per site (Online Methods and Supplementary Note) from the sample population in the 34-Mb target region. Among these SNPs, 53,707 (44.1%) had not been previously reported (dbSNP, version 129), and 80% of these newly identified SNPs had MAF < 5%. We assessed the accuracy of our genotype calls on a randomly chosen subsample of the new SNPs and found inconsistent genotypes for 9% of the very rare SNPs (defined as MAF < 0.02) but no inconsistent genotypes for the more common SNPs, indicating a low overall false positive rate (Online Methods, Supplementary Note and Supplementary Tables 2 and 3). The false negative rate of SNPs with MAF > 0.02 was estimated to be 5.1% using SNPs that had been genotyped in the HapMap European CEU population (Supplementary Note and Supplementary Fig. 1). In all, 25,275 synonymous and 27,806 non-synonymous coding SNPs were identified (Supplementary Tables 4 and 5), of which 22,216 (42.6%) were new.

Our large population sample provided the opportunity to characterize the impact of natural selection on protein coding genes, (Supplementary Note, Supplementary Tables 6 and 7) something that has been difficult in previous studies of Europeans due to limitations based on either the number of genes analyzed or the sample size. The distribution of allele frequencies can reveal signatures of natural selection, but an accurate estimation of allele frequencies using next generation sequencing data is challenging due to high error rates and varying coverage. Relying on such genotypes to compute allele frequencies thus leads to biased estimates, as even the best method for calling genotypes results in a biased measure of population genetic variability.

We circumvented this pitfall by developing an unbiased (minimum variance) estimator of allele frequencies, which directly relies on the nucleotide reads observed in each individual exome and accounts for error rates and coverage variation. Simulations and stringent quality thresholds indicated that our estimator was reliable for derived alleles with frequencies of over 2% in our dataset (Online Methods and Supplementary Fig. 2). According to above genotyping validations results, we observed the false positive results only in SNPs with MAF < 0.02 (though the false positive rate in this frequency range was still <10%). Hence, we only included SNPs with MAF > 0.02 in our subsequent analyses to ensure the validity of our conclusions.

To quantify the effect of selection on deleterious mutations, we compared the distribution of allele frequencies among non-synonymous (that is, amino acid changing) and synonymous cSNPs (Fig. 1a). Synonymous cSNPs closely followed the distribution expected in the absence of natural selection. In contrast, non-synonymous cSNPs showed a much larger proportion of low frequency alleles, indicative of a strong purifying selection, showing that a large proportion of these mutations were likely to be deleterious. We observed a notable 1.8-fold excess in the proportion of non-synonymous to synonymous cSNPs for alleles with low (2–4%) frequencies. Although this excess is not incompatible with findings in previous studies, our study included a larger sample size and suggests that the excess of low frequency non-synonymous mutations predominantly comes from very rare mutations (that is, mutations with frequencies <4%) and not from higher frequency mutations. To further demonstrate that the pattern we observed was due to the presence of deleterious mutations and not to sequencing artifacts, we categorized sites with non-synonymous cSNPs according to their conservation across species. As expected, substitutions on very conserved sites tended to segregate at much lower frequencies than mutations on less conserved sites (Fig. 1b). We obtained similar results when classifying non-synonymous SNPs on the basis of the amino acid change induced (Supplementary Fig. 2b).

We assessed the amount of selection required to explain the observed differences between the distributions of synonymous and non-synonymous cSNPs. Our estimator, γ, was an effective population-scaled selection coefficient that may differ from actual selection coefficients, as we ignored demographic effects and linkage among sites. However, as the synonymous cSNPs closely followed the neutral expectation in our data (Fig. 1a), we expect our method to provide estimates that are in agreement with previous work, which also ignored linkage but relied on demographic models. We assumed that γ followed a mixture distribution, with a proportion (k) of non-synonymous cSNPs being neutral (γ = 0) and other non-synonymous cSNPs having γ values that follow a gamma distribution with parameters α and β (Online Methods). We estimated α = 4, β = 0.09 and k = 0.2, which indicated a larger proportion of weakly deleterious and mildly deleterious (~80%) mutations than did previous estimates (54%) which led to the discovery of a larger proportion of low frequency non-synonymous mutations than was previously identified in smaller
The sequence data has been deposited in the NCBI-12-P2-43-ethetics. Likewise, biased gene conversion from A and T to G and C has been sites alone have no effect on the frequency spectrum. It has been observed that changes in the mutation rate alone explain the pattern observed, as changes in the mutation rate have a stronger effect on the frequency spectrum in non-synonymous sites, including positive selection and higher excess of rare non-synonymous mutations on the X chromo, than on autosomes. Depending on the true dominance relationships between the X chromosome and autosomes, with a higher excess of these mutations present in the X chromosome (Fig. 1c). The X chromosome is well known to have less variation than autosomes. Many factors may contribute to this effect, including mutation rate variation, but the lower effective population size on the X chromosome may be the main underlying cause. Another contributing factor may be the presence of recessive deleterious mutations exposed to selection on the X chromosome in males. If selected mutations are recessive, selection will have a stronger effect on the X chromosome than on autosomes. Depending on the true dominance relationships and distribution of selection coefficients, this effect is also a possible explanation for the excess of rare non-synonymous mutations relative to synonymous mutations on the X chromosome observed in this study. Negative selection acting on recessive mutations may prevent more of these mutations from reaching intermediate frequencies on the X chromosome than on the autosomes, leading to a relatively higher excess of rare non-synonymous mutations on the X chromosome. However, other selective factors may affect the frequency spectrum in non-synonymous sites, including positive selection and Hill-Robertson effects. Explanations based solely on mutation rate variation, such as a higher or lower mutation rate in males, cannot alone explain the pattern observed, as changes in the mutation rate alone have no effect on the frequency spectrum.

Processes unrelated to selection may also affect the observed frequencies of alleles. Methylation leads to hypermutability in CpG sites, which would promote recurrent mutations at the same sites. Likewise, biased gene conversion from A and T to G and C has been proposed to be common in mammals, especially in regions with high recombination rates. This type of gene conversion would result in a segregation distortion that favors the transmission of C or G alleles. If this effect is strong and pervasive, the site frequency spectrum of mutations from A and T to G and C may differ from that of other mutations. Using fourfold degenerate sites, we compared the frequency distributions for sites potentially affected by CpG methylation or gene conversion versus unaffected sites (Fig. 1d and Online Methods). The distributions we obtained were not significantly different from each other (P > 0.05; Mann-Whitney U test), indicating that, when considered at a genome-wide scale, neither CpG mutation nor biased gene conversion had a strong effect on the frequency spectrum.

We report, to our knowledge, the largest dataset of directly sequenced human exomes published to date. Although we only sequenced the exome regions and the sequence coverage per individual was not adequate to ascertain genotypes for each individual with high confidence, our data can be used for accurate estimation of the allele frequencies of cSNPs with MAF > 0.02. These data are therefore useful for studying the allele frequency spectrum, as well as other population genetics patterns in this sample cohort. We find that coding regions harbor a larger proportion of low-frequency deleterious mutations than previously anticipated. Our analyses indicate that most of these mutations are recessive, which partly explains their segregation in humans despite strong purifying selection. Our observations are also consistent with recent claims that only a very small proportion of the heritable variation associated with common polygenic traits has been identified in association studies. Based on our findings, we support the idea that much of the heritable variation affecting fitness is caused by low-frequency mutations, which are often overlooked in studies based on genotyping rather than resequencing. Further, we demonstrate that the use of next-generation sequencing is an important tool in population genetics studies. Future analyses of non-coding regions and ethnically diverse samples will help build a complete picture of human genomic variation and an understanding of the interaction between genetic drift, mutation, recombination and selection in the human genome.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

**Accession codes.** The sequence data has been deposited in the NCBI Short Read Archive with accession number SRA009884.

*Note: Supplementary information is available on the Nature Genetics website.*

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AUTHOR CONTRIBUTIONS

COMPETING FINANCIAL INTERESTS
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ONLINE METHODS

Sample acquisition and exon sequencing. Genomic DNA was purified from blood leukocytes from 200 individuals of Danish nationality. Exome capture was performed on a NimbleGen 2.1M HD array (Roche). Exon-enriched DNA libraries were then subjected to a secondary library construction for Illumina GA sequencing and were sequenced by the Illumina Genome Analyzer II platform following the manufacturer’s instructions (Supplementary Note).

Genotype calling and SNP calling. SOAPaligner superscript24,25 was used to align the sequencing reads to the NCBI human genome reference assembly (build 36.3). The likelihood of possible genotypes at each site in each individual was calculated using SOAPmap superscript26. For each site, the likelihoods from all sample individuals were then integrated using a heuristic formula to give a score that measured the confidence to ascertain SNPs. Highly confident SNPs were then extracted for subsequent analysis. A randomly selected subset of SNPs that were not deposited in the dbSNP database (v129) was genotyped using a Sequenom iPLEX array for validation (Supplementary Note).

Estimation of allele frequencies. Our aim was to obtain unbiased estimates of allele frequencies. This is difficult to do using SNP calling, as most procedures for SNP calling tend to lead to a deficiency or an excess of rare variants depending on how conservative the applied method is superscript12. Although it is possible to correct the sample frequency spectrum obtained on the basis of SNP calling statistically superscript12, we instead chose to pursue an arguably more direct approach for estimating allele frequencies for each SNP. To do this, we first eliminated all reads with Q score < 20. The mismatch rate in these reads was 0.41%. We then determined which two nucleotides were most common among A, C, T and G. We let the set of these nucleotides be S, meaning if there were 400 As, 42 Cs, 13 Ts and 9 Gs, then S = {A, C}. We then eliminated all reads that were not elements of S. This was done under the assumption that all SNPs are truly diallelic. Because we eventually used a frequency cutoff of 2%, ignoring the third and fourth most common bases had no effect on our inferences.

We let ni be the number of reads of the minor allele in S in individual i. We let the total number of reads in S in individual i be nips. Then we calculated

\[ p_i = \frac{n_{iC}}{n_{iC} + n_{iT}}, \]

where \( n_{iC} \) is the true number of reads that should be a C, but due to sequencing errors, we observed \( n_{iC} \) instead. Because we assume sites are biallelic, \( n_{iC} = n_{iT} \). If we divide by \( n_{iC} \), we get

\[ p_i = \frac{n_{iT}}{n_{iC} + n_{iT}} \]  
for \( i = 1, 2, \ldots, k \). This is an error-corrected estimate of the allele frequency in individual \( i \), obtained as the solution for \( p_i \) to the equation. Note that it is possible for \( p_i \) to be negative (for example, if \( n_i = 0 \)). The parameter \( e \) is the error rate and is considered a fixed parameter. On the basis of previous analyses, we assume \( e = 0.004 \). This estimate is, if anything, an overestimate because it represents the error rate between the major and minor allele only. For example, suppose the nucleotide read at a site for an individual is A, then due to a sequencing error, an A can be changed to a T, C or G. We assume sites are diallelic, therefore we only consider one change, which implies that 0.4% is an overestimate of the error rate (as a 1% per-read-base mismatch rate divided by 3 is less than 0.4%). We then calculate

\[ w_i = \frac{2n_{iT}}{n_{iT} + 1} \]  
(see derivation below), the inverse of the variance of \( p_i \) (up to a scalar). Note that \( w_i \) lies between (0,2), takes the value 0 when there are no reads, 1 when there is 1 read (when there was only one chromosome sampled) and 2 as the number of reads tends to infinity (corresponding to certainty that the 2 chromosomes have been sampled).

The estimate of the minor allele frequency is then calculated as

\[ \hat{p} = \max \left\{ \frac{0 \sum_{i=1}^{k} p_i w_i}{\sum_{i=1}^{k} w_i} \right\} \]

This estimate of allele frequencies was combined among autosomal sites with population coverage of at least 600-fold (the coverage thresholds for X and Y chromosome sites were 438(X) and 162(Y)) in accordance with the number of X chromosomes and closely approximated the true distribution of parameter values; we conducted several simulation studies. The method clearly estimated the distribution accurately. The performance of the method was aided by the use of a large sample size. For smaller sample sizes, the use of a distribution of estimates to approximate the estimate of a distribution would lead to a larger discrepancy between the estimated and true distribution.

Derivation of weights, \( w_i \). Without loss of generality, we assumed that we have two alleles A (minor allele) and T (major allele). Let

\[ Y_i = I_1 + \ldots + I_n_{IT} \]

which is the proportion of reads with allele A, where \( I_i \) takes the value 1 when the read is an A and 0 when the read is not an A.

We now need to find the variance of \( Y_i \),

\[ \text{Var}(Y_i) = \frac{\text{Var}(I_1 + \ldots + I_n_{IT})}{n_{IT}^2} \]

\[ = \frac{1}{n_{IT}^2} \text{Var}(I_1 + \ldots + I_n_{IT}) \]

\[ = \frac{1}{n_{IT}^2} \left( \text{Var}(I_1) + \ldots + \text{Var}(I_n_{IT}) + 2 \sum_{i<j} \text{Cov}(I_i, I_j) \right) \]

The variance terms, \( \text{Var}(I_i) \), are easily computed and are equal to

\[ \text{Var}(I_i) = p_i(1 - p_i) \]

where \( p_i \) is the population probability of observing a read with an A. The covariance term,

\[ \text{Cov}(I_i, I_j) = E(I_iI_j) - E(I_i)E(I_j) \]

can also be computed assuming Hardy-Weinberg equilibrium.

The second term is \( E(I_i)E(I_j) = p_i^2 \). To compute the first term, let \( M = I_i^2 \).

Then by the law of total probability,

\[ P(M = 1) = P(M = 1 | AA)P(AA) + P(M = 1 | AT)P(AT) + P(M = 1 | TT)P(TT) \]

So the probability \( P(M = 1) \) depends on the genotype of the individual. Assuming H-W equilibrium,

\[ P(M = 1) = p_t^2 + \frac{1}{4}(2p_t(1 - p_t)) + 0 \]

\[ = p_t^2 + \frac{1}{2}p_t(1 - p_t) \]

Therefore,

\[ \text{Var}(I_1 + \ldots + I_n_{IT}) = n_{IT}p_t(1 - p_t) + 2 \left( \frac{p_t(1 - p_t)}{2} + \frac{1}{2}p_t(1 - p_t) - p_t^2 \right) \]

\[ = p_t^2 \]
α-SNP detection for massively parallel whole-genome resequencing. SOAP2: an improved ultrafast tool for short read alignment.

Supplementary Figure 3 compares the gamma distributions of selective effects obtained here and previously, indicating that we detected proportionally more moderate and mildly deleterious mutations. This is likely due to the fact that we had a larger sample size, which facilitated the detection of very rare mildly deleterious variants.

SNP annotation. We polarized SNPs into ancestral and derived variants using the multiz8way alignments available from UCSC (see URLs). For all detected SNPs covered with a level 1 chimpanzee net alignment, we extracted the corresponding chimpanzee and macaque nucleotide from these alignments. When chimpanzee and macaque shared the same nucleotide at a given SNP position, the human variant shared with these two other species was called ancestral. SNPs with no inferred ancestral state were discarded from all downstream analyses. Using RefSeq annotation, we classified SNPs into five functional categories (non-synonymous, synonymous, substitutions at fourfold degenerate sites, 5' untranslated region and 3' untranslated region) according to their genomic location and their expected effect on the encoded gene products. We also classified SNPs into substitutions that are potentially affected by gene conversion (AT→GC) or not (A→T and G→C). Similarly, we distinguished between SNPs at CpG sites that were potentially due to deamination of methylated cytosines (CpG→TpG, CpG→CpA) or not (CpG→ApG, CpG→GpG, CpG→CpC and CpG→CpA).

We assessed the potential deleterious effect of non-synonymous SNPs using placental mammal conservation scores computed by Phylop (available at UCSC, see URLs). As an alternative approach, we used the physicochemical distances between pairs of amino acids as previously described.

Estimation of selection coefficients. We fit a model for the density of non-synonymous mutations, f(x), observed across the whole genome as:

\[ f(x) = k(l/x) + (1-k) f_{sel}(x, -\gamma) \text{Gamma}(\gamma; \alpha, \beta) d\gamma \]

where the first term is a contribution from a fraction, k, of the sites having a neutral distribution, and the second term describes negative selection acting on the remaining sites. The first term in the integral, f_{sel}, is the theoretical limiting density of frequencies of mutations in a large population (the mean density of the PRF). The negative selection parameter \( \gamma \) follows a gamma distribution with parameters \( \alpha \) and \( \beta \). f(x) is evaluated for 50 points evenly spaced between frequencies 0.02 and 0.98, and the counts of non-synonymous mutations were calculated in the same range in 50 equally spaced bins. The model was then fit using least squares. We defined a grid of points from \( k = 0, \ldots, 1 \) in steps of 0.02, \( \alpha = 2, \ldots, 6 \) in steps of 0.2 and \( \beta = 0.05, \ldots, 0.77 \) in steps of 0.04. We then evaluated the least-squares function over the grid. We found the best fitting parameter values to be \( k = 0.2 \), \( \alpha = 4 \) and \( \beta = 0.09 \), with a least-squares cost of 0.28. This indicated that most of the sites were under negative selection, with a mean selection parameter of \( (\alpha/\beta) = 45 \). Previous estimates based on the same model\(^9\) found \( k = 0.23 \), \( (\alpha/\beta) = 816 \) (with \( \alpha = 1.02 \)) and predicted that 54% of all mutations were either weakly or moderately deleterious. Using the same assumptions regarding effective population size and definitions of weakly deleterious and mildly deleterious mutations, we found (by integrating our estimated distribution over values corresponding to \( 2N_s = 1 \) to \( 2N_s = 1000 \)) that the proportion of such mutations was 80%.