Imputation of Rare Variants in Next-Generation Association Studies

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Abstract
The role of rare variants has become a focus in the search for association with complex traits. Imputation is a powerful and cost-efficient tool to access variants that have not been directly typed, but there are several challenges when imputing rare variants, most notably reference panel selection. Extensions to rare variant association tests to incorporate genotype uncertainty from imputation are discussed, as well as the use of imputed low-frequency and rare variants in the study of population isolates.

Introduction
Many disease associations with common variants have been detected via hundreds of genome-wide association studies (GWAS), yet these variants are only able to explain a small fraction of the heritable component of disease. Moreover, in the search for genetic susceptibility to disease, the analysis focus has shifted from common variants to the identification and association analysis of low-frequency and rare variants. Typically, common variants are defined to have minor allele frequency (MAF) >0.05, while low-frequency variants have MAF 0.01–0.05, and rare variants are those with MAF <0.01.

In focusing on variants with low allele frequencies, the challenge of being able to accurately genotype them arises. The majority of genotype arrays were designed with common variants in mind, but with the changing focus to less common variants, high-density genotyping arrays (e.g., Illumina Omni 2.5M) have been designed enabling lower MAF variants to be genotyped. The most accurate way of accessing rare variants is through next-generation sequencing, which is not easily available for large samples due to its high cost. Imputation is a cost-effective alternative that is able to estimate genotypes that are not directly typed, and relies on the availability of a dense reference panel. Genetic correlations are then extrapolated from the reference panel to the study sample to approximate the unobserved genotypes, thereby increasing the number of variants available for association testing, and, consequently, providing a higher chance of detecting true associations [1–3]. Among the standard imputation tools, IMPUTE [4] and MACH [5] generally have better performance when estimating missing genotypes at rare polymorphisms, since they take into account all available markers and all available haplotypes, but this also makes them substantially more computationally intensive [6]. Computational time for imputation may be decreased by first pre-phasing the data, which has been demonstrated to be effective at low-frequency SNPs; for each individual within the GWAS sample, the haplotypes are first statisti-
cally estimated and then missing genotypes are imputed into these estimated haplotypes [7].

There are several considerations when imputing low-frequency and rare variants, which are due to challenges that are not encountered when dealing with higher MAF SNPs. Low-frequency and rare variants are more difficult to tag than common SNPs, which often results in the poor performance of imputation, as illustrated by Pei et al. [8], Huang et al. [9] and Sung et al. [10]. However, there are ways of selecting a reference panel so as to maximize the number of low-frequency/rare variants imputed with high quality. Reference panel selection is discussed in the next section, followed by approaches to accessing non-common variants by making use of higher density data for a portion of the study sample in conjunction with imputation. Next, association analysis methods for imputed low-frequency and rare variants are discussed. The analysis of rare variants has difficulty in itself due to their low MAF, but the additional uncertainty from imputed genotypes adds to the challenge. There are several methods developed for rare variant association analysis that have been extended to handle imputed rare genotypes as well. The approaches to imputing rare variants in population isolates are subsequently discussed. Low haplotype diversity within population isolates makes it possible to use only a relatively small sequenced sample to impute variants in the remaining population. Studies in which rare variant imputation has been incorporated are then summarized, followed by a discussion on future directions in the imputation of rare variants. A flow chart of the steps taken in the use of imputation in a rare variant association analysis is provided in figure 1.

**Reference Panel Selection**

The objective of the International HapMap Project was to catalog common sequence variants and determine their frequencies as well as correlation patterns across the genome [11]. HapMap is composed of samples taken from populations with African, Asian, and European ancestry as well as recently admixed populations from the Americas, and includes variants that are common in at least one of the study populations. There are currently 3 phases of HapMap: Phase 3 data are formed from genotyping 1.6 million common SNPs in 1,184 reference individuals from 11 global populations, and sequencing ten 100-kb regions in a subset of 692 individuals. In HapMap 3, it was confirmed that, even among closely related populations, lower-frequency variation is less shared than common variation [12].

In keeping with its design, the HapMap reference panel has good coverage down to MAF 0.05 and sparser coverage at lower MAFs [13]. This limitation may be surpassed by making use of the 1000 Genomes Project [14]. One of the key aims of the 1000 Genomes Project was to identify more than 95% of variants with MAF as low as 0.01 across the genome and from 0.001 to 0.005 in gene regions, by sequencing 2,500 individuals, thereby providing the scientific community with a catalogue of variation that is more extensive than the HapMap. Consequently, in comparison to HapMap-based imputations, many more variants, particularly rare and low-frequency variants, can be imputed by using the 1000 Genomes Project data reference panel.

A marker imputability database has been assembled (http://www.unc.edu/~yunmli/imputability.html) to provide imputation accuracy information for 1000 Genomes Project variants across four major continental groups and multiple genotyping platforms (pers. commun., Yun Li). A leave-one-out imputation procedure was applied to the 1000 Genomes Project samples, as a means of calculating the imputation accuracy of each variant, and markers that were absent from a given platform were treated as untyped.

With a focus on imputation performance in Europeans, the HapMap 2 and HapMap 3 reference panels were
compared by Jostins et al. [15], considering a variety of population combinations within each HapMap phase. The use of all populations in HapMap 3 as a reference panel was found to yield highly accurate imputation of low-frequency/rare variants close to the accuracy attained in common variant imputation. Further, if the ancestry-matched sample is not sufficiently large, then the imputation accuracy of low-frequency variants can increase with the addition of haplotypes from other populations. On the other hand, when there is a sufficiently large number of reference haplotypes from the relevant population, increasing the diversity of the reference panel may not improve imputation accuracy. This is also a consequence of a theoretical investigation of how reference panel properties affect imputation accuracy via a coalescent model [16]. The study showed that there is greater imputation accuracy from a modestly sized reference panel composed of individuals from the same population as the target haplotype than from a larger panel consisting of a different population.

Consistent with Jostins et al. [15], Sung et al. [10] showed that, for low-frequency variants, although the CEU panel from the 1000 Genomes Project (60 individuals, June 2010 release) and the EUR panel from the 1000 Genomes Project (283 individuals, August 2010 release) both achieved higher imputation performance than HapMap 2-based imputation, the larger and more diverse EUR 1000 Genomes Project panel was most successful. They found that 1000 Genomes Project-EUR-based imputations yielded 4 times as many successfully imputed low-frequency SNPs than those based on the HapMap-CEU panel, and the increase was 8-fold for rare variants.

Primarily, evaluation of imputation performance has focused on European populations. However, there is a growing interest in non-European populations, including African and those that are recently admixed, such as Hispanic. African populations are challenging to impute due to their lower levels of LD, and they were found to have the lowest imputation accuracy among 29 populations in the Human Genome Diversity Project [9]. African-American populations have the additional challenge of admixture, since it complicates the choice of an ideal reference panel.

Considering that the genomes of Hispanics, African-Americans, and other recently admixed populations may be partitioned into long genomic regions arising from one of a few ancestral populations, a method has been developed to construct a tailor-made reference population for each region of the genome [17]. Upon application of this method to select a weighted haplotype reference panel based on a set of populations, any of the current imputation methods may then be employed. In assessing the use of weighted haplotype panels, the largest gain in accuracy was attained in regions of low LD; when used with IMPUTE v2 on a Hispanic dataset, the total number of SNPs with imputed correlation >0.6 increased from 76% (IMPUTE v2) to 83% (IMPUTE v2 with weighted haplotype panels). Moreover, with this same dataset, the error rate was found to be consistently lower across the range of MAFs (from <0.05 to common) when the tailor-made haplotype panels were used.

It was shown by Howie et al. [18] that cosmopolitan reference panels increase imputation accuracy at low MAF variants for a variety of populations, including African. In their comparison of imputing a Gambian population based on a Gambian-specific panel versus HapMap 3, which does not include Gambians, they found that the much smaller Gambian panel (∼200 chromosomes) yielded higher accuracy than HapMap, which included around 800 chromosomes of African ancestry. However, the difference in imputation accuracy was not large, and smallest at low-frequency variants. Furthermore, for MAF 0.01–0.03, HapMap-based imputation had the highest accuracy, indicating that low-frequency alleles that are poorly represented in the Gambian panel could be captured by an ancestrally inclusive, non-specific reference panel. Upon using the HapMap 3 reference panel in addition to the Gambian panel, the imputation accuracy increased over that of the single Gambian reference panel. These results suggest that incorporating haplotypes from all available reference panels achieves the highest accuracy in African populations, among others.

In Sung et al. [19], three imputation strategies are evaluated for African-American populations, and a comparison is made between the HapMap 2 and 1000 Genomes Project as reference panels, in an attempt to overcome the issues in imputing such populations. The union strategy, which uses a panel consisting of SNPs polymorphic in either CEU or YRI, was found to have the best performance. The other strategies considered were intersection, which uses a reference panel composed of SNPs polymorphic in both CEU and YRI panels, and merge, which involves merging the results of two separate imputations, one YRI-based and one CEU-based, where each imputation uses SNPs polymorphic in the single panel. In line with the European imputation results of Sung et al. [10], they found that the 1000 Genomes Project-based imputations provided approximately 8 times as many well-imputed
rare and low-frequency variants as those that were HapMap-based. Furthermore, the accuracy attained in the 1000 Genomes Project-based imputations was similar to that of the HapMap-based imputations. It is anticipated that imputation performance for African-Americans will improve with versions of the 1000 Genomes Project data that include larger samples.

An alternative, but more costly option, to employing a publicly available reference panel is to sequence a subset of the study sample. Li et al. [3] sequenced (with a median depth of 27×) the exons and flanking regions of 202 genes that are current or prospective drug targets in over 14,000 samples including population-based and case collections for 12 diseases. Among the sequenced samples, 8,865 samples also had GWAS data available on either the Affymetrix 500K, Affymetrix 6.0, or Illumina 550K platforms. For each platform, the authors first partitioned these samples into a study set of size 270 and a reference set that ranged in size from 100 to 3,713, then used the reference data to impute the genotypes in the GWAS data. In order to assess imputation performance using the various reference sets and platforms, they compared the imputed genotypes with the genotypes derived from the high-depth sequence data, which enables evaluation for variants with MAF <0.01, due to the high quality of the derived genotype calls. It was illustrated by Li et al. [3] that for very rare variants (MAF <0.005) the imputation performance depends highly on the reference panel size, and such variants are not likely to be imputable if the reference panel does not contain at least 1,200 individuals. The authors suggest the routine use of imputation for common and moderately rare variants, based on their finding that for reference panel sizes of at least 1,200 subjects, approximately 40% of variants with 0.005 < MAF < 0.05 and almost all common SNPs will be imputable. The results also indicate that Illumina 550K tended to result in higher imputation quality than Affymetrix 500K, particularly for low-frequency variants, where the proportion of variants that could be imputed well was 78 and 57% for Illumina 550K and Affymetrix 500K, respectively, when a reference panel of 3,713 individuals was used.

In simulation studies, Mägi et al. [20] compared the power of a regression-based collapsing method within four approaches to accessing rare variants: re-sequencing, genotyping the variants present in a reference panel, imputation, and genome-wide genotype data. They demonstrated that the power attained in analyzing genome-wide genotype data imputed up to a high-density reference panel is comparable to that of the much more costly re-sequencing gold standard.

A further approach is to impute based on both the 1000 Genomes Project data and a sequenced subset of the data. In Fridley et al. [21], an evaluation is made on the imputation performance of sequencing a subset of a sample to be used as a reference panel, as well as employing the 1000 Genomes Project data as a reference panel. They concluded that it is best to select the largest and most diverse reference panel for sequencing, and to maximize the number of markers that are genotyped for all subjects, based on their finding that imputation accuracy is influenced by both the number of markers genotyped and the reference sample size. The 1000 Genomes Project sequencing data may be used in conjunction with a reference panel based on a subset of the sample that is sequenced, in order to maximize the number of variants, particularly rare and low-frequency variants, that could be imputed. Fridley et al. [21] noted that a 1000 Genomes Project-based imputation without a sequenced subset from the sample may not give sufficient representation of the genetic diversity in affected individuals.

The ongoing UK10K Project (http://www.uk10k.org/) has the aim of identifying low-frequency and rare variants and their associations with disease and quantitative traits by sequencing 10,000 individuals primarily from the UK. Within the sample, the exomes of 6,000 cases with extreme obesity, rare disorders and neurodevelopmental disease are high-depth sequenced, while 4,000 individuals from population-based cohorts with deep phenotype data are genome-wide sequenced at low depth (average 6×). The low-depth whole-genome sequenced data will aid in imputation of previously genotyped cohorts and increase the power in such case-control studies. Moreover, the 6,000 high-depth sequenced exomes of extreme phenotypes will result in high power to detect rare variant associations with the diseases in-
Accessing Rare Variants

There are two general issues that need to be considered when testing for associations with rare variants. First, it needs to be decided how to access the rare variants. This may be done via sequencing, genotyping variants present in a reference panel, or imputation; the methods are listed in order of decreasing cost. Second, a method for detection of association needs to be chosen.

In accessing rare variants, an option is a two-platform design, where all individuals are genotyped, and a small subset is also genotyped on a more costly, denser platform or sequenced, as considered by Fridley et al. [21] and discussed in the previous section. This design was further explored by Sampson et al. [23], who imputed missing genotypes of individuals genotyped only on a low-density array (Illumina OmniExpress; >700,000 SNPs) by using a reference set that included both high-density genotype data (Illumina Omni2.5-Quad; approximately 2.5 million SNPs) in conjunction with public datasets. They demonstrated that even with high-density genotyping a subset as small as 100 individuals, it may be possible to observe more than 80% of the detectable associations from directly genotyping all individuals. This is 5–10% more than when imputation is based only on a public reference set and there is no high-density genotyping. However, the authors also illustrate that if rare variants have relative risks that are significantly larger than what has been observed for common variants, then the relative proportion would likely be lower. With a higher relative risk for variants with MAF <0.05, the total proportion of associations detected by the two-platform design increases, but the proportion relative to the number of detected associations when all individuals are genotyped decreases. This relative decrease is a consequence of low imputation accuracy for low MAF variants, so that genotyping only a subset of the individuals and then imputing performs poorer than directly genotyping everyone.

Along the lines of the two-platform design, there is the option of the more costly two-stage design in which a subset of the sample is sequenced to discover variants, and identified variants are then genotyped in the remaining sample, rather than genotyped on a higher density array. The portion chosen to be sequenced may be a balanced number of cases and controls, or only cases. Use of the former strategy may detect protective variants for the disease phenotype with higher probability [24], while the latter approach may have higher power for detecting causal variant associations. In a comparison of the two study designs with varying sequenced subset size, Liu and Leal [25] showed that when relatively few samples are sequenced (e.g. 400 samples from a study of 3,000 with equal cases and controls), the cases-only strategy is more powerful, but this advantage grows smaller as the sequenced subset increases in size.

When portions of both cases and controls are selected for sequencing, the sequence and genotype data may be combined by meta-analysis methods. However, if only some cases are sequenced, integrating the genotyped and sequenced data directly, by comparing aggregated variant frequencies between cases and controls, leads to inflated type I errors in the test for rare variant associations [26]. Liu and Leal [25] discussed and compared various methods that have been developed to correct for this bias, and introduced a likelihood-based method, SEQCHIP, which makes an adjustment such that the variant genotypes found from sequencing only cases are corrected to follow the same distribution as the genotyped samples. These SEQCHIP data may then be used with any rare variant association test that incorporates genotype uncertainty. The authors illustrated that SEQCHIP-integrated data yielded well-controlled type I errors for rare variant association tests, as well as consistently higher power than other data integration methods.

The Analysis of Imputed Rare Variants

It is often difficult to detect associations with rare/low-frequency variants by applying single variant tests, due to their low frequency. However, imputation can lead to a boost in power at these variants [2]. The software SNPTEST v2 (https://mathgen.stats.ox.ac.uk/genetics_software/snptest/old/snptest_v2.3.0.html) implements single variant tests that account for uncertainty in imputed genotypes [4].

A computationally efficient method to test for single SNP associations in GWAS, when complete or imputed genotype data are available and also when including related individuals, is introduced by Zhou and Stephens [27] as Genome-Wide Efficient Mixed-Model Association (GEMMA). GEMMA efficiently performs exact calculations, rather than approximations, and is shown to provide numerically identical results to the widely used exact method Efficient Mixed-Model Association
Population Isolates and Rare Variant Imputation

Population isolates are of interest due to their lower phenotypic, environmental, and genetic heterogeneity, which allows easier detection of variants that are rare in frequency in the outbred populations from which the isolates diverged. Increases in genetic homogeneity and rare variant allele frequency tend to occur in populations resulting from the expansion of a relatively small number of founders who formed a population bottleneck, or from random genetic drift. Thus, variants that are rare elsewhere may have a higher frequency in isolated populations.

A long-range phasing approach was introduced by Kong et al. [39] to infer haplotype phase irrespective of
the availability of parental genotypes, provided that a sufficiently large sample from the isolated population has been genotyped. This method elicits latent information from such data by recognizing the usefulness of moderately to distantly related individuals, rather than focusing only on closely related pairs. The close genetic relationships in isolated founder populations are further taken advantage of in the genotype phasing algorithm developed by Palin et al. [40], referred to as systematic long-range phasing (SLRP). SLRP uses a model that explicitly considers identity-by-descent (IBD) segments and models multiple individuals sharing a haplotype.

High levels of relatedness in isolated founder populations are also capitalized in the imputation algorithm of Uricchio et al. [41] by using the IBD information in a small number of sequenced founder individuals for imputation of a much larger number of founders with only genome-wide SNP genotypes. The authors demonstrate that across the full range of allele frequencies, with high accuracy and low missing information, their method can phase and impute genotypes identified from sequencing in members of this founder population (South Dakota Hutterites).

With a focus on rare variant inference in the whole-genome analysis of a homogeneous isolate population, Gusev et al. [42] showed that low-pass sequencing together with multi-sample calling is able to yield a 2- to 3-fold gain in accurate variant detection over independent calling. The authors studied an isolated cohort from the Pacific island of Kosrae, Micronesia, and estimated that at least 60% of the cohort’s 3,000 genomes could be inferred from a full panel of 40 sequenced individuals. However, they cautioned that this is a unique cohort, and in a more diverse isolated population it is likely that a lower proportion of the population genome could be inferred from sequencing a sample of size 40; in an isolated cohort of Ashkenazi Jewish origin only 23% of the genome could be inferred using 40 sequenced individuals.

Studies Employing Rare Variant Imputation

In this section, we summarize some representative examples of studies published to date that have searched for associations with low-frequency/rare variants by using imputation.

In a proof-of-principle for next-generation association study design [43], capitalizing on the unique characteristics of population isolates and combining whole-genome sequencing with imputation, Holm et al. [44] identified a missense mutation associated with sick sinus syndrome in MYH6 through GWAS and imputation of 1000 Genomes Project variants, followed by whole-genome sequencing for refinement and genotyping for validation purposes in an Icelandic cohort. The variant is not present in the HapMap or 1000 Genomes Project data and was not identified in any of the European-descent controls investigated.

In the study by Day-Williams et al. [45], a 1000 Genomes Project-based imputation into a GWAS dataset of osteoarthritis cases and controls from the UK was used to detect a previously unidentified risk locus on chromosome 13. Through large-scale replication, the authors established a robust association with SNPs in MCF2L (risk allele frequency of approximately 0.07).

A susceptibility locus for venous thrombosis on chromosome 11p11.2 was detected, and it was found that the signal was attributed to a rare functional variant, which is approximately 1 Mb away from the locus and has a MAF of approximately 0.03 [46]. This locus discovery arose by using the 1000 Genomes Project (August 2010 release) reference dataset for imputation of two GWAS. Previous imputation based on HapMap Phase 2 had missed the signal.

In an imputation analysis of ADAMTSL3 as a candidate gene for schizophrenia, Dow et al. [47] identified several rare variants that may have a functional impact in terms of protein function, gene regulation and splicing, although verification is required. The authors sequenced 92 schizophrenia cases from a previous GWAS [48], and imputed the data using European-descent samples from both the HapMap Phase 3 and 1000 Genomes Project data as reference panels. In their sequencing study they did not detect any additional variants that were in strong LD with the previously identified GWAS SNPs, and the same previously identified variants were found to have the strongest association signals in the imputation analysis.

In the only example to use rare variant aggregate tests with imputed data to date, Mági et al. [20] used GRAN-VIL to analyze 14,000 cases of 7 common complex diseases and 3,000 shared controls from the WTCCC [49], imputed using multiple populations from the 1000 Genomes Project Phase 1 reference panel (June 2011 interim release), to re-assess the evidence for association with rare variants. Genome-wide significant evidence of an association between an accumulation of rare variants and decreased risk of coronary artery disease was identified in PRDM10, while 10 genes (9 risk-increasing, 1 protective) within the MHC exhibited genome-wide significant rare variant associations with type 1 diabetes.
Discussion

The analysis of rare variants is a relatively new field that has grown exponentially with the development of analysis methods and access approaches, but to date there remain few published studies that employ rare variant association methods. However, it is anticipated that there will soon be an increase in the practical use of locus-based tests designed for rare variants, as a greater understanding of the methods and challenges are addressed. General difficulties in rare variant analysis include choice of a significance threshold for locus-based tests, as well as selection of regions for analysis. A few of the possible region choices are sliding windows of a fixed size, sliding windows of a fixed number of rare variants, or functional units.

Additional challenges include the functional annotation of non-coding rare variants, population stratification, and family-based analysis. The prediction of molecular function at non-coding variants is more difficult than at coding variants, and there are databases consisting of known non-coding variant that affect phenotype, but these are not of use for novel variants, as is often the case for rare variants [32]. There are existing methods that can adjust for confounding of population structure, but under certain conditions such methods have been demonstrated to be ineffective for rare variants [50]. Specifically, when the distribution of non-genetic risk is concentrated into a small, sharply defined region(s), rare variants were shown to have a tendency towards stronger stratification that is systematically different from that for common variants, so that the usual corrections used in the standard GWAS setting are not effective. The authors describe three ways in which such a scenario may occur:

1. highly patchy localized environmental exposure;
2. systematic measurement bias at a recruitment centre, and
3. local variation in recruitment policy or misclassification rates.

Family-based designs are more likely to be enriched for rare variants, since if one parent possesses a rare minor allele, half of the children are expected to carry it as well [51]. Two adaptive weighting methods for rare variant association tests in family-based data have been recently proposed for sequenced data with quantitative traits by Fang et al. [52]. Through simulation studies, they showed that their methods are powerful, even when there are different directions of effect, and robust to population stratification. There is currently no extension to imputed data, but as with other rare variant methods, a quick extension is to replace genotype scores by dosages. However, it is unknown how this change to the genotype accuracy will affect the power of the test.

Methodology for testing rare variant associations continues to grow, and many challenges still remain. Imputation has been proven to be a useful tool in accessing non-common variants, but careful selection of a reference panel is essential to achieve highly accurate results. It has become more common for studies to take advantage of imputation to access low-frequency/rare variants for association testing, and as more studies combine such data with powerful rare variant analysis tools, additional pieces of the missing heritability problem are expected to be filled.

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