A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data

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ABSTRACT

Motivation: Most existing methods for DNA sequence analysis rely on accurate sequences or genotypes. However, in applications of the next-generation sequencing (NGS), accurate genotypes may not be easily obtained (e.g. multi-sample low-coverage sequencing or somatic mutation discovery). These applications press for the development of new methods for analyzing sequence data with uncertainty.

Results: We present a statistical framework for calling SNPs, discovering somatic mutations, inferring population genetical parameters, and performing association tests directly based on sequencing data without explicit genotyping or linkage-based imputation. On real data, we demonstrate that our method achieves comparable accuracy to alternative methods for estimating site allele count, for inferring allele frequency spectrum and for association mapping. We also highlight the necessity of using symmetric data sets for finding somatic mutations and confirm that for discovering rare events, mismapping is frequently the leading source of errors.

Availability: http://samtools.sourceforge.net

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1 INTRODUCTION

The 1000 Genomes Project [1000 Genomes Project Consortium 2010] sets an excellent example on how to design a sequencing project to get the maximum output pertinent to human populations. An important lesson from this project is to sequence many human samples at relatively low coverage instead of a few samples at high coverage. We adopt this strategy because with higher coverage, we will mostly reconfirm information from other reads, but with more samples, we will be able to reduce the sampling fluctuations, gain power on variants present in multiple samples and get access to many more rare variants. On the other hand, sequencing errors counteract the power in variant calling, which necessitates a minimum coverage. The optimal balancing point is broadly regarded to be in the 2–6 fold range per sample [Le and Durbin 2010; Li et al. 2011], depending on the sequencing error rate, level of linkage disequilibrium (LD) and the purpose of the project.

A major concern with this design is that at 2–6 fold coverage per sample, non-reference alleles may not always be covered by sequence reads, especially at heterozygous loci. Calling variants from each individual and then combining the calls usually yield poor results. The preferred strategy is to enhance the power of variant discovery by jointly considering all samples [Le and Durbin 2010; Li et al. 2011; Depristo et al. 2011; Nielsen et al. 2011]. This strategy largely solves the variant discovery problem, but acquiring accurate genotypes for each individual remains unsolved. Without accurate genotypes, most of previous methods (e.g. testing Hardy-Weinberg equilibrium (HWE) and association mapping) would not work.

To reuse the rich methods developed for genotyping data, the 1000 Genomes Project proposes to impute genotypes utilizing LD across loci [Li et al. 2009b; Browning and Yu 2009; Howie et al. 2009; Li et al. 2010a]. Suppose at a site \( A \), one sample has a low coverage. If some samples at \( A \) have high coverage and there exists a site \( B \) that is linked with \( A \) and has sufficient sequence support, we can transfer information across sites and between individuals, and thus make a reliable inference for the low-coverage sample at \( A \). The overall genotype accuracy can be greatly improved.

However, imputation is not without potential concerns. Firstly, imputation cannot be used to infer the regional allele frequency spectrum (AFS) because imputation as of now can only be applied to candidate variant sites, while we need to consider non-variants to infer AFS. Secondly, the effectiveness of imputation depends on the pattern of LD, which may lead to potential bias in population genetical inferences. Thirdly, the current imputation algorithms are slow. For a thousand samples, the fastest algorithm may be slower than read mapping algorithms, which is frequently the bottleneck of analyzing NGS data (Hyun Min Kang, personal communication). More samples and the use of more accurate imputation algorithms will be even slower.

These potential concerns make us reconsider if imputation is always preferred. We notice that we perform imputation mainly to reuse the methods developed for genotyping data, but would it be possible to derive new methods to solve classical medical and population genetical problems without precise genotypes?

Another application of NGS that requires genotype data is to discover somatic mutations or germline mutations between a few related samples [Ley et al. 2008; Mardis et al. 2009; Shah et al. 2009; Pleasance et al. 2010a,b; Roach et al. 2010; Conrad et al. 2011]. For such an application, samples are often sequenced to high coverage. Although it is not hard to achieve an error rate one per 100,000 bases [Bentley et al. 2008], mutations occur at a much lower rate, typically of the order of \( 10^{-6} \) or even \( 10^{-7} \). Naively calling genotypes and then comparing samples frequently would not work well [Ajay et al. 2011], because subtle uncertainty in genotypes may lead to a bulk of errors. From another angle, however, when discovering rare mutations, we only care about the difference between samples. Genotypes are just a way of measuring the difference. Is it really necessary to go through the genotype calling step?
This article explores the answer to these questions. We will show in the following how to compute various statistics directly from sequencing data without knowing genotypes. We will also evaluate our methods on real data.

2 METHODS

This section presents the precise equations on how to infer various statistics such as the genotype frequency and AFS, and to perform various statistical test such as testing HWE and associations. Some of these equations have already been described in the existing literature, but for theoretical completeness, we give the equations using our notations. The last subsection reviews the existing methods and summarizes the differences between them, as well as between ours and existing formulation.

In the Methods section, we suppose there are $n$ individuals with the $i$-th individual having $m_i$ ploidy. At a site, the sequence data for the $i$-th individual is represented as $d_i$ and the genotype is $g_i$ which is an integer in $[0, m_i]$, equal to the number of reference alleles in the individual.

Table 1 gives notations common across this Methods section. The detailed derivation of the equations in this article is presented in an online document [http://bit.ly/stmath](http://bit.ly/stmath).

### Table 1. Common notations

| Symbol | Description |
|--------|-------------|
| $n$    | Number of samples |
| $m_i$  | Ploidy of the $i$-th sample ($1 \leq i \leq n$) |
| $M$    | Total number of chromosomes in samples: $M = \sum_i m_i$ |
| $d_i$  | Sequencing data (bases and qualities) for the $i$-th sample |
| $g_i$  | Genotype (the number of reference alleles) of the $i$-th sample ($0 \leq g_i \leq m_i$) |
| $\phi_k$ | Probability of observing $k$ reference alleles ($\sum_k \phi_k = 1$) |
| $\Pr[A]$ | Probability of an event $A$ |
| $\mathcal{L}_i(\theta)$ | Likelihood function for the $i$-th sample: $\mathcal{L}_i(\theta) = \Pr\{d_i|\theta\}$ |

1 In this article, we only consider biallelic variants.

2.1 Assumptions

2.1.1 Site independence We assume data at different sites are independent. This may not be true in real data because sequencing and mapping are context dependent; when there is an insertions or deletion (INDEL) error or INDEL polymorphism, sites nearby are also correlated in mapping are context dependent; when there is an insertions or deletion in real data because sequencing and dropping the less evident types if present. Thus at any site we see at most two types of nucleotides. This treatment is not optimal, but sufficient in practice.

Assume at a site there are $k$ reads. Without losing generality, let the first $l$ bases ($l \leq k$) be identical to the reference and the rest be different. The error probability of the $j$-th read base is $\epsilon_j$. Assuming error independency, we can derive that

$$
\mathcal{L}(g) = \frac{1}{m^l} \prod_{j=1}^{l} \left[ (m-g)\epsilon_j + g(1-\epsilon_j) \right] \prod_{j=l+1}^{k} \left[ (m-g)(1-\epsilon_j) + g\epsilon_j \right]
$$

(2)

where $m$ is the ploidy.

2.1.3 Biallelic variants We assume all variants are biallelic. In the human population, the fraction of triallelic SNPs is about 0.2% [Hodgkinson and Eyre-Walker 2010]. The biallele assumption does not have a big impact to the modeling of SNPs, though it may have a bigger impact to the modeling of INDELs at microsatellites.

2.2 Computing genotype likelihoods

For one sample at a site, the sequencing data $d$ is composed of an array of bases on sequencing reads plus their base qualities. As we only consider biallelic variants, we may focus on the two most evident types of nucleotides and drop the less evident types if present. Thus at any site we see at most two types of nucleotides. This treatment is not optimal, but sufficient in practice.

Assume at a site there are $k$ reads. Without losing generality, let the first $l$ bases ($l \leq k$) be identical to the reference and the rest be different. The error probability of the $j$-th read base is $\epsilon_j$. Assuming error independency, we can derive that

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$$

(2)

where $m$ is the ploidy.

2.3 Inferences from multiple samples

2.3.1 Estimating the site allele frequency In this section we estimate the per-site reference allele frequency $\psi$. For the $i$-th sample, let $m_i$ be the the ploidy, $g_i$ the genotype and $d_i$ the sequencing data. Assuming Hardy-Weinberg equilibrium (HWE), we can compute the likelihood of $\psi$.

$$
\mathcal{L}(\psi) = \prod_{i=1}^{n} \prod_{g_i=1}^{m_i} \Pr\{d_i, g_i|\psi\} = \prod_{i=1}^{n} \sum_{g_i=0}^{m_i} \mathcal{L}_i(g; m_i, \psi) \tag{3}
$$

where $\mathcal{L}_i(g; m_i, \psi)$ is computed by Eq. (2) and

$$
f(g; m, \psi) = \binom{m}{g} \psi^g (1-\psi)^{m-g} \tag{4}
$$

is the probability mass function of the binomial distribution $\text{Binom}(m, \psi)$.

Knowing the likelihood of $\psi$, we may numerically find the max-likelihood estimate with, for example, Brent’s method [Brent 1973]. An alternative approach is to infer using an expectation-maximization algorithm (EM), regarding the sample genotypes as missing data. Given we know the estimate $\psi^{(t)}$ at the $t$-th iteration, the estimate at the $(t+1)$-th iteration is

$$
\psi^{(t+1)} = \frac{1}{M} \sum_{i=1}^{n} \sum_{g} \frac{\mathcal{L}_i(g; f(g; m_i, \psi^{(t)}))}{\mathcal{L}_i(g; f(g; m_i, \psi^{(t)}))} \tag{5}
$$

where $M = \sum_i m_i$ is the total number of chromosomes in samples.

When the signal from the data is strong, or equivalently for each $i$, one of $\mathcal{L}_i(g)$ is much larger than others, the EM algorithm converges faster than the direct numerical solution using Brent’s method. However, when the signal from the data is weak, numerical method may converge faster than EM [Kim et al. 2011]. In implementation, we apply 10 rounds of EM iterations. If the estimate does not converge after 10 rounds, we switch to Brent’s method.

2.3.2 Estimating the genotype frequencies In this section, we assume all samples have the same ploidy: $m = m_2 = \cdots = m_n$, and aim to estimate $\xi_g$, the frequency of genotype $g$. The likelihood of $\{\xi_0, \ldots, \xi_m\}$ is:

$$
\mathcal{L}(\xi_0, \ldots, \xi_m) = \prod_{i=0}^{m} \mathcal{L}_i(g) \xi_g \tag{6}
$$

with the constraint $\sum_g \xi_g = 1$. The EM iteration equation is

$$
\xi_g^{(t+1)} = \frac{1}{n} \sum_{i=1}^{n} \frac{\mathcal{L}_i(g; \xi_g^{(t)})}{\sum_{g'} \mathcal{L}_i(g'; \xi_g^{(t)})} \tag{7}
$$

An important application of genotype frequencies is to test HWE for diploid samples ($m=2$). When genotypes are known, we can perform
a 1-degree $\chi^2$ test. This approach would not work for sequencing data as it does not account for the uncertainty in genotypes, especially when the average read depth of each individual is low. A proper solution is to perform a likelihood-ratio test (LRT). The test statistic is

$$D_k = -2 \log \frac{L(\hat{\psi})}{L(\hat{z}_0, \hat{z}_1, \hat{z}_2)} = -2 \log \frac{L((1 - \hat{\psi})^2, 2\hat{\psi}(1 - \hat{\psi}), \hat{\psi}^2)}{L(\hat{z}_0, \hat{z}_1, \hat{z}_2)}$$

(8)

where

$$\hat{\psi} = \arg \max \psi L(\psi)$$

(9)

is the max-likelihood estimate of the site allele frequency and similarly $\hat{z}_0$, $\hat{z}_1$ and $\hat{z}_2$ are the max-likelihood estimate of the genotype frequencies. Because $L(\hat{\psi})$ has one degree of freedom and $L(\hat{z}_0, \hat{z}_1, \hat{z}_2)$ has two degrees of freedom, the $D_k$ statistic approximately follows the 1-degree $\chi^2$ distribution. For genotype data, $D_k$ approaches the standard HWE test statistic computed from a 3-by-2 contingency table.

2.3.3 Estimating haplotype frequencies between loci In this section, we assume all samples are diploid. Given $k$ loci, let $h = (h_1, \ldots, h_k)$ be a haplotype where $h_j$ equals 1 if the allele at the $j$-th locus is identical to the reference, and 0 otherwise. Let $n_k$ be the frequency of haplotype $h$ satisfying $\sum h_k = 1$, where

$$n_k = \sum_{i=1}^{n} \sum_{j=1}^{n} \sum_{k=1}^{n} \eta(h_1, \ldots, h_k)$$

When sample genotypes are all certain, this EM iteration is reduced to the standard EM for estimating haplotype frequencies using genotype data [Excoffier and Slatkin, 1995].

The time complexity of computing Eq. (10) is $O(n \cdot d^4)$ and thus it is impractical to estimate the haplotype frequency for many loci jointly. A typical use of Eq. (10) is to measure linkage disequilibrium (LD) between two loci.

2.3.4 Testing associations Suppose we divide samples into two groups of size $n_1$ and $n_2$, respectively, and want to test if group 1 significantly differs from group 2. One possible test statistic could be [Kim et al., 2010, 2011]

$$D_{a1} = -2 \log \frac{L(\hat{\psi})}{L(\hat{\psi})[\hat{\psi}(1 - \hat{\psi})][\hat{\psi}^2]}$$

(11)

where $\hat{\psi}$ is the max-likelihood estimate of the site allele frequency of all samples (Eq. [9]), and $\hat{\psi}[1]$ and $\hat{\psi}[2]$ are the estimates of allele frequency in group 1 and group 2, respectively. Under the null hypothesis, $D_{a1}$ approximately follows the 1-degree $\chi^2$ distribution.

A potential concern with the $D_{a1}$ statistic is that the computation of $L(\hat{\psi})$ assumes HWE. When HWE is violated, false positives may arise [Nielsen et al., 2011]. For diploid samples, a safer statistic is

$$D_{a2} = -2 \log \frac{L(\hat{\eta}, \hat{z}_0, \hat{z}_1, \hat{z}_2)}{L(\hat{\eta}[1], \hat{\eta}[2], \hat{\eta}[3], \hat{\eta}[4], \hat{\eta}[5], \hat{\eta}[6])}$$

(12)

which in principle follows the 2-degree $\chi^2$ distribution under the null hypothesis. However, when both cases and controls are in HWE, the degree of freedom is reduced and this statistic is underestimated.

We have not found a powerful test statistic robust to HWE violation. For practical applications, we propose to take the $P$-value computed with $D_{a1}$, while filtering candidates having a low $D_{a2}$ to reduce false positives caused by HWE violation (see Results).

2.3.5 Estimating the number of non-reference alleles In this section we use the term site reference allele count to refer to the number of reference alleles at one single site. Allele count is a discrete number while allele frequency is contiguous.

For convenience, define random vector $\bar{G} = (G_1, \ldots, G_n)$ to be a genotype configuration, and $X = \sum G_i$ to be the site reference allele count in all the samples. Assuming HWE, we have

$$\Pr \{G_i = g|X = k\} = \delta_{h_s, x_0}(g) \prod_{i=1}^{n} \left(\frac{m_i}{4k}\right)$$

(13)

where $s_0(g) = \sum g_i$ is the total number of reference alleles in a genotype configuration, $g_i$, and $\delta_{h_s}$ is the Kronecker delta function which equals 1 if $k = 1$ and equals 0 otherwise. The likelihood of allele count is

$$L(k) = \Pr \{\bar{d}, X = k\} = \frac{1}{k^m} \sum_{g_1=0}^{m} \cdots \sum_{g_n=0}^{m} \delta_{h_s, x_0}(g) \prod_{i=1}^{n} \left(\frac{m_i}{4k}\right) L_i(g_i)$$

(14)

for $0 \leq l \leq \sum_{i=1}^{n} m_i$, and $z_{jl} = 0$ otherwise. $z_{jl}$ can be calculated iteratively with

$$z_{jl} = \sum_{g_1=0}^{m} \cdots \sum_{g_l=0}^{m} \delta_{h_s, x_0}(g) \prod_{i=1}^{l} \left(\frac{m_i}{4k}\right) L_i(g_i)$$

(15)

starting from $z_{00} = 1$. Comparing the definition of $z_{nl}$ and Eq. (15), we know that

$$L(k) = \frac{n_k}{\binom{4k}{k}}$$

(16)

which computes the likelihood of the allele count.

Although the computation of the likelihood function $L(k)$ is more complex than of $L(\psi)$, $L(k)$ is discrete, which is more convenient to maximize or sum over. This likelihood function establishes the foundation of the Bayesian inference.

2.3.6 Numerical stability of the allele count estimation When computing $z_{jl}$ with Eq. (15), floating point underflow may occur given large $j$. A numerically stable approach is to compute $y_{jl} = z_{jl}/\binom{M}{l}$ instead, where $M_j = \sum_{i=1}^{l} m_i$. Thus

$$L(k) = n_k$$

(17)

and by replacing $z_{jl}$ with $y_{jk}/\binom{M}{l}$ in Eq. (15), we can derive:

$$y_{jk} = \sum_{l=0}^{j} \prod_{i=0}^{l-1} \left(\frac{k-l}{M_j-i}\right) \sum_{g_j=0}^{m_j} y_{j-1,l-g_j} \cdot \binom{m_j}{g_j} L_j(g_j)$$

(18)

$$\cdot \sum_{l=0}^{j} \prod_{i=0}^{l-1} \left(\frac{M_j-i}{k-l}\right)$$

However, we note that $y_{jk}$ may decrease exponentially with increasing $j$. Floating point underflow may still occur. An even better solution is to rescale $y_{jl}$ for each $j$, similar to the treatment of the forward algorithm for Hidden Markov Models [Durbin et al., 1998]. In practical implementation, we compute

$$\tilde{y}_{jl} = \frac{y_{jl}}{\prod_{j'=1}^{l} t_j}$$

(19)

where $t_j$ is chosen such that $\sum_{j} y_{jl} = 1$.

As an alternative implementation note, most $y_{jl}$ are close to zero and thus $y_{nk}$ can be computed in a band rather than in a triangle. This may dramatically speed up the computation of the likelihood.
### 2.3.7 Calling variants

In variant calling, we have a strong prior knowledge that at most of sites all samples are homzygous to the reference. To utilize the prior knowledge, we may adopt a Bayesian inference for variant calling. Let $φ_{k}$, $k = 1, \ldots, M$, be the probability of seeing $k$ reference alleles among $M$ chromosomes/haplotypes. For convenience, define $Φ = \{φ_k\}$, which is in fact the sample allele frequency spectrum (AFS) for $M$ chromosomes. Recall that $X$ is the number of reference alleles in the samples. The posterior of $X$ is

$$Pr\{X = k|d, Φ\} = \phi_k Pr\{d|X = k\}/\sum\phi_i Pr\{d|X = i\} = \phi_k L(k)/\sum\phi_i L(i)$$

(20)

where $L(k)$ is defined by Eq. [14] and computed by Eq. [17]. In variant calling, we define variant quality as

$$Q_{\text{var}} = -10\log_{10} Pr\{X = M|d, Φ\}$$

and call the site as a variant if $Q_{\text{var}}$ is large enough. Because in deriving $L(k)$, we do not require the ploidy of each sample to be the same. The variant calling method described here are in theory applicable to pooled resequencing with unequal pool sizes.

### 2.3.8 Estimating the sample allele frequency spectrum (AFS)

For variant calling, we typically take the Wright-Fisher AFS as the prior. We can also estimate the sample AFS with the maximum-likelihood inference when the Wright-Fisher prior deviates from the data.

Suppose we have $L$ sites of interest and we want to estimate the frequency spectrum across these sites. Let $X_a$, $a = 1, \ldots, L$, be a random variable representing the number of reference alleles at site $a$. We can use an EM algorithm to find $Φ$ that maximizes $Pr\{\theta|d, Φ\}$, the probability of data across all samples and all sites conditional on AFS. The iteration equation is

$$φ_k^{(t+1)} = \frac{1}{L} \sum_a Pr\{X_a = k|d, Φ^{(t)}\}$$

(21)

We call this method of estimating AFS as EM-AFS. Alternatively, we may also acquire the max-likelihood estimate of the allele count at each site using Eq. [15]. The normalized histogram of these counts gives the AFS. We call this method as site-AFS. We will compare the two methods in the Results section.

### 2.4 Discovering somatic and germline mutations

One of the key goals in cancer resequencing is to identify the somatic mutations between a normal-tumor sample pair (Kosson 2010), which can be achieved by computing a likelihood ratio. Given a pair of samples, the following likelihood ratio is an informative score:

$$D_p = -2\log L[\hat{g}]/L[\hat{g}^{(1)}]$$

(22)

where $L[\cdot]$ is computed by Eq. [3]. $\hat{g}$ maximizes $L[\hat{g}]$, and similarly $\hat{g}^{(1)}$ and $\hat{g}^{(2)}$ maximize $L[\hat{g}^{(1)}]$ and $L[\hat{g}^{(2)}]$, respectively. When this stands, we have:

$$L[\hat{g}]/L[\hat{g}^{(2)}]$$

(23)

and then we can prove:

$$D_p = 2\log \left\{ L[\hat{g}^{(1)}]/L[\hat{g}^{(2)}] \right\}$$

This equation has an intuitive interpretation: we are certain about a candidate somatic mutation only if both genotypes in both samples are clearly better than other possible genotypes.

A natural extension to discovering somatic mutations is to discover de novo and somatic mutations in a family trio (Conrad et al. 2011). To identify such mutations, we may compute the maximum likelihoods of genotype configurations without the family constraint and with the constraint, and then take the ratio between the two resulting likelihoods. The larger the ratio, the more confident the mutation. More exactly, the likelihood ratio is:

$$D_i = -2\log \frac{\max_{(g_c, g_f, g_m)\in G} \{L_c(g_c) L_f(g_f) L_m(g_m)\}}{\max L_c(g_c) \cdot \max L_f(g_f) \cdot \max L_m(g_m)}$$

(24)

where $L_c(g_c)$, $L_f(g_f)$ and $L_m(g_m)$ are the child, father and mother genotype likelihoods respectively, and $G$ is the set of genotype configurations satisfying the Mendelian inheritance.

Although most of the derivation in this article assumes variants are bialelic, we drop this assumption in the implementation for methods described in this subsection. We have observed false somatic/germline mutations caused by the mismodeling of triallelic variants (Mark Depristo, personal communication). The biallelic assumption may lead to false positives.

### 2.5 Working with diploid multi-allelic sites

Suppose at a site there are $p$ alleles. The site frequency of allele $h$ being $ψ_h$ with $\sum_h ψ_h = 1$. If we assume the site is under the Hardy-Weinberg equilibrium, the likelihood function of $(ψ_1, \ldots, ψ_p)$ is:

$$L(ψ_1, \ldots, ψ_p) = \prod_{i=1}^{p} L_i(h_i, h_i') ψ_h ψ_{h'}$$

(25)

where $(h_i, h_i')$ represents a pair of unordered integers, or a diploid genotype. The EM iteration equation can be derived as:

$$ψ_{h'}^{(t+1)} = \frac{1}{n} \sum_{i=1}^{n} \sum_{h_i'} L((h_i, h_i')) ψ_h^{(t)} ψ_{h'}^{(t)}$$

(26)

To test whether a site is multi-allelic, we may compute the likelihood ratio

$$D_{m1} = -2\log \frac{L(0, 1)}{L(ψ_1, ψ_2, ψ_3)}$$

(27)

as is proposed by Kim et al. (2010).

### 2.6 Related works

During SNP calling, Thunder (Li et al. 2011) and gffMultiB (http://bit.ly/gffmultib) compute the site allele frequency by numerically maximizing the likelihood (Eq. [2]). Genome Analysis Toolkit (GATK; Depristo et al. 2011) infers the frequency with EM (Eq. [5]), infers the frequency with both the numerical and the EM algorithms. Li et al. (2009b) derived an alternative method to estimate the site allele frequency, which is not covered in this article. SeqEM (Martin et al. 2010) estimates the genotype frequency using EM (Eq. [10]) with a different parameterization. Le and Durbin (2010) derived Eq. [16]. The conclusion is correct, but the derivation is not rigorous: the binomial coefficient in Eq. [13] was left out. Yi et al. (2010) came to a similar set of equations to Eq. [15] and [20], but the prior is taken from the estimated site allele frequency. To the best of our knowledge, Kim et al. (2010) is the first to use genotype likelihood based LRT to compute P-value of associations (Eq. [17]) with more thorough evaluation in a recent paper (Kim et al. 2011; Nielsen et al. 2011) further proposed to test associations with a score test (Schaad et al. 2002). Except Kim et al. (2010), all the previous works focus on diploid samples, while many equations in this article can be in theory applied to multi-pleidy samples and pooled samples.

In this article, our contribution includes testing HWE, estimating haplotype frequency, the proposal of two-degree association test, a simple but effective model for discovering somatic mutations, the rigorous derivation and numerically stable implementation of a discrete allele count estimator, and an EM algorithm for inferring AFS.
3 RESULTS

3.1 Implementation
Most of equations for diploid samples \( m = 2 \) have been implemented in the SAMTools software package (Li et al., 2009a), which is distributed under the MIT open source license, free to both academic and commercial uses. The exact Eq. (7)–(9) have also been implemented in GATK as the default SNP calling model.

The SAMTools package consists of two key components, samtools and bcftools. The former computes the genotype likelihood \( L(g) \) using an improved version of Eq. (2) which considers error dependencies; the latter component calls variants and infers various statistics described in this article. To clearly separate the two steps, we designed a new Binary variant call format (BCF), which is the binary representation of the variant call format (VCF; Danecek et al., 2011) and is more compact and much faster to process than VCF. On real data, computing genotype likelihoods especially for INDELs is typically 10 times slower than variant calling. The separation of genotype likelihood computation and subsequent inferences enhances the flexibility and improves the efficiency for inferring AFS. Bcftools also directly works with VCF files, but is less efficient than with BCF files.

Table 2 shows how VCF information tags generated by SAMTools are related to the equations in this article. We refer to the SAMTools manual page for detailed description.

### Table 2. SAMTools specific VCF information

| INFO   | Equation | Description |
|--------|----------|-------------|
| AF1    |          | Non-reference site allele frequency |
| G3     |          | Diploid genotype frequency |
| HWE    |          | P-value of Hardy-Weinberg equilibrium |
| NEIR   |          | Neighboring \( r^2 \) linkage disequilibrium statistic |
| LRT    |          | 1-degree association test P-value |
| LRT2   |          | 2-degree association test P-value |
| AC1    |          | Non-reference site allele count |
| AC2    |          | Prob. of the site being poly. among samples |
| CLR    |          | Log likelihood ratio score for \textit{de novo} mutations |

1 Tag at the VCF additional information field (INFO)
2 Related, though not exact, equations for computing the values

### 3.2 Inferring the allele count
We downloaded the chromosome 20 alignments of 49 Pilot-1 CEU samples sequenced by the 1000 Genomes Project using the Illumina technology only. We called the SNPs with SAMTools and imputed the genotypes with Beagle under the default settings. At 32,522 sites genotyped using the Omni genotyping chip and polymorphic in the 49 samples, the root-mean-square deviation (RMSD) between the allele count acquired from Omni genotypes and the estimate using Eq. (16) equals 3.7, the same as the RMSD between the Omni and the Beagle-imputed genotypes. Not surprisingly, imputed genotypes are more accurate when there is a tightly linked SNP nearby, while the imputation-free estimate is less affected (Fig. 1).

However, on the unreleased European data from the 1000 Genomes Project consisting of 670 samples, Beagle imputation is better than our imputation-free method (RMSD(imput)=12.7; RMSD(imput-free)=15.0). We conjure that this is because with more samples, it is more frequent for two samples to share a long haplotype. The LD plays a more important role in counteracting the lack of coverage. Nonetheless, we should beware that sites selected on the Omni genotyping chip may not be a good representative of all SNPs. For example, for the sites on the Omni chip, only 8% of SNPs do not have a nearby SNP with \( r^2 > 0.05 \) in a 20-SNP window (the ‘nearby SNPs’ include all SNPs discovered in the 670 samples), but this percentage is increased to 30% for all SNPs. The large fraction of unlinked SNPs might hurt the accuracy of imputation based methods.

We have also evaluated our method on an unpublished target resequencing data set consisting of about 2000 samples (Haiman et al., personal communication). The imputation based method does not perform well (RMSD(imput)=54.8; RMSD(imput-free)=42.5), probably due to the lack of linked SNPs around fragmented target regions.

### 3.3 Inferring the allele frequency spectrum
To evaluate the accuracy of the estimated allele frequency spectrum (AFS), we compared the AFS obtained from the low-coverage data produced by the 1000 Genomes Project and from the high-coverage data released by Complete Genomics (http://bit.ly/m7LzvF). Fig. 2 reveals that we can infer a fairly accurate AFS using the EM-AFS method with 3-fold coverage per sample. On the other hand, the site-AFS estimate is less stable, though the overall trend looks right. To estimate properties across multiple sites, summing over the posterior distribution using EM-AFS is more appropriate.

### 3.4 Performing association test
To evaluate the performance of the association test statistics \( D_{a1} \) (Eq. (11)), we constructed a perfect negative control using the 1000 Genomes data and derived the empirical distribution of \( D_{a1} \). We expect to see no associations. Fig. 3 shows that \( D_{a1} \) largely follows
the 1-degree likelihood ratio test (Eq. 11) and the other by the canonical 1-degree LRT based on Beagle imputed genotypes; in the right, the 2-degree LRT. Closer investigation reveals that although DNA used in the two data sets was originated from the same individual, somatic mutations in cell lines, which is of the order of 1,000 per diploid genome (Conrad et al., 2011), may be present. If the cell lines used in two studies have greatly diverged, we might see up to a dozen somatic mutations on chromosome 20. This time with a threshold $D_p \geq 30$ and a maximum depth filter 150, we identified 667 single-base differences between the two data sets, far more than our expectation. Again we sought to reduce mapping errors by remapping reads with BWA-SW to the 1000 Genomes Project phase-2 reference genome. The number of differences between the HiSeq and the old Illumina data quickly drops to 33. If we further filter out clustered SNPs using a 100bp window, 13 potential differences are left, 2% of the initial candidates. This exercise again proves that mismapping is the leading source of errors.

To see if the simple likelihood ratio (Eq. 22) is comparable to more sophisticated methods, we briefly tried SomaticSniper (Larson et al., 2011) on our data. With a somatic score cutoff 65, which is about 30 in the ‘2 log’ scale as in $D_p$, SomaticSniper identified 1,826 differences. SAMtools called fewer because it limits the mapping quality of reads with excessive mismatches and applies base alignment quality (Li, 2011) to fix alignment errors around INDELs. With the two features switched off, SAMtools called 1,696 differences, half of which overlap the differences found by SomaticSniper. Calls unique to one method tend to have a mutation score close to the threshold.

### 4 DISCUSSIONS

We have proposed a statistical framework for SNP calling as well as analyzing sequencing data but without explicitly calling SNPs or their genotypes. With this framework, we can discover somatic and germline mutations with appropriate input data, efficiently estimate site allele frequency, allele frequency spectrum and
linkage disequilibrium, and test Hardy-Weinberg equilibrium and association. On real data, we have demonstrated that our method is able to achieve comparable accuracy to the best alternative methods. We have also extensively evaluated the performance of our method on several unpublished data sets and got sensible results. Thus we conclude that useful information can be obtained directly from sequencing data without SNP calling or imputation.

Here we also want to emphasize a few findings in our evaluation of the methods. Firstly, we confirmed that imputation is a viable method for transferring our knowledges on genotyping data to low-coverage sequencing data. It is likely to have higher accuracy than our method given homogeneous whole-genome data consisting of many samples. Nonetheless, we showed that the accuracy of imputation depends on the LD nearby, which has long been speculated but without direct evidence from real data until our work. Secondly, our proposed EM-AFS method is able to accurately estimate AFS from low-coverage sequencing data. It is more appropriate than estimating the site frequency separately and then doing a histogram. Thirdly, we observed that violation of HWE may cause false positives in association mapping with the one-degree likelihood ratio test (Kim et al. 2011). A two-degree likelihood ratio test is a conservative way to avoid such an artifact. At last, we highlighted the importance of using data of similar characteristics in the discovery of somatic mutations. We also want to put a particular emphasis on the necessity of controlling mapping errors when looking for very rare events such as somatic mutations, germline mutations and RNA editing. It may be necessary to use two distinct mapping algorithms to call variants and then take the intersection.

Frequently we require to know the exact DNA sequences or genotypes only to estimate parameters or compute statistics. In these cases, the sequences and genotypes are just intermediate results. When the sequence itself is uncertain, mostly due to the uncertainty in sequencing and mapping, it may sometimes be preferred to directly work with the uncertain sequence which may carry more information than an arbitrarily ascertained sequence. We have showed that many population genetical parameters and statistical tests can be adapted to work on uncertain sequences, and believe more existing methods can be adapted in a similar manner. Knowing the exact sequence is convenient, but not always indispensable.

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