

SCarborSNV

Efficient Phylogeny-aware Single Nucleotide Variant Detection for
Single Cells

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Abstract

Ongoing somatic mutations during cancer development lead to genetically distinct subclonal populations of cells within a tumour, each with a distinct subset of acquired mutations. These subclonal populations genetically diverge as new mutations occur and are subject to Darwinian selection pressures. This leads to a complex intra-tumour heterogeneity, the subclonal architecture of which is important for understanding cancer evolution and developing individualised therapies. Some approaches have used bulk DNA sequencing coupled with advanced clustering techniques to attempt to tease out this structure. Recent advances in single-cell DNA sequencing (SCS), however, have allowed new approaches such as Monovar and SCIΦ to examine this heterogeneity directly, despite the inherent low quality of the SCS data. We here present a new probabilistic algorithm, SCarborSNV, which we expect will efficiently call point mutations using aligned SCS sequencing data from a sample of multiple cells. After calling candidate variant loci using a detailed prior, SCarborSNV uses a neighbour joining algorithm to reconstruct a phylogeny which is used to genotype individual cells. We will compare SCarborSNV with existing methods on simulated and real data and expect to show that SCarborSNV performs competitively in calling single cell genotypes.

1 Introduction

Inferring the various genetic mutations present in a sample of phylogenetically related single cells is a complicated task, especially in light of the low quality data generated by SCS methods. As such, previous methods have pooled information from across all cells in a sample to more accurately distinguish between bona fide mutations and noise. The commonly used Monovar algorithm pools the information from across cells by marginalizing on the variable total alternate allele count across all cells at each locus, using statistical methods derived from population genetics models [1]. A more recent approach, SciΦ, has found further success by leveraging the fact that the sampled cells are phylogenetically related. By first gaining some understanding of this ancestral relationship, SciΦ has been able to improve calling accuracy with similar algorithmic complexity [2].

The SCarborSNV algorithm presented here intends to follow on in the direction pioneered by SciΦ, using phylogenetic inference to distinguish between real mutations and SCS noise. SCarborSNV will attempt to provide similar calling accuracy with asymptotically improved complexity in the number of cells sampled, from $O(m^3)$ to $O(m^2)$.

1.1 Cells, Mutation and Intra-tumour Heterogeneity

While individual organisms are mostly genetically homogeneous, mutagenic factors can cause the genotypes of cells within an organism to diverge from the germline. These somatic mutations are often inconsequential, but when they cause a cell to gain “an autonomous will to divide, this aberrant uncontrolled cell division [can create] masses of tissue (tumors) that invade organs and destroy normal tissue.” [3]. Such a collection of rapidly dividing cells will share all the genetic mutations of their ancestors, including both germline mutations and the set of somatic mutations that caused the expansion. In the context of tumor evolution, these mutations are known as clonal (or truncal) mutations as they are common to all cells in the clone: the set of all cells clonally descended from the original mutant cell. As the cells continue to divide rapidly, some cells in the clone will undergo further somatic mutations, known as subclonal mutations. Any subset of tumor cells descended from such a further mutated cell (all containing the subclonal mutation) are referred to as a subclone.

After the tumor has had sufficient time to develop further mutations, the genetic landscape of the cells within the tumor will be one of heirarchical subclones; the mutations within any given cell will correspond with its specific ancestry of subclonal mutations within the tumor. At the time of biopsy, there are often a large number of different subclones, each with a unique ancestry and thus a unique set of clonal and subclonal mutations. This genetic variation between cells in the tumor is called intra-tumor heterogeneity, and is of

great importance for understanding tumor development, metastasis and treatment [4].

Since the genetic mutations by themselves leave few or no clues as to when in the tumor's history they occurred, resolving this subclonal structure can be challenging.

1.2 High Throughput Sequencing and Bulk Approaches

While other approaches have tried to read long sequences of DNA (or coDNA)(?) directly, the high throughput sequencing (HTS) or next generation sequencing (NGS) approach is to amplify the original sequence many times before breaking it down into smaller overlapping subsequences and reassembling the short reads therefrom in silico. This shotgun approach of HTS has proved far faster and cheaper than other methods, as well as being highly accurate (depending on how many short reads cover a given site). It is possible to reconstruct de novo sequences by piecing together the overlaps of the short reads, but for known organisms the reads can be quickly aligned to a reference genome, with some flexibility to account for mutations and variations.

The standard approach for HTS is to extract the DNA from many thousands or millions of cells, amplify the region to be sequenced a few times (e.g. by PCR) and finally align the short reads. This will hereafter be referred to as bulk sequencing, as the genetic material is extracted from a bulk tissue sample (as opposed to from individual cells). Bulk sequencing is very common practice, requires no specialized technology to separate cells, and achieves a high level of calling accuracy with a high read depth (many reads mapped to each locus). Because of this bulk HTS data has been used to reconstruct tumor phylogenies, for example by ClonEvol. At each locus, the proportion of reads containing mutant reads is used as an ersatz for the number of mutant cells. These values can be used to discover clusters of mutations with similar frequencies, and potential orderings or phylogenies of these mutations can be evaluated based on how likely it is each cluster descends from the others.

Bulk analyses have provided insight into ITH, however the results are difficult to verify and ????? smaller subclones may be missed. TODO TODO TODO. To resolve ITH more directly, the genotype of individual cells must be examined.

1.3 Single Cell Sequencing

As opposed to bulk sequencing, where the DNA from a large number of cells is aggregated before amplification and sequencing, single cell sequencing separates out single cells for individual examination and sequencing. After separating out individual cells, there are two methods of analyzing the genetic material therefrom: single cell RNA sequencing (scRNA-seq) and single cell DNA sequencing (SCS), each method

with its own advantages and disadvantages. The more common scRNA-seq first forms co-DNA from the mRNA present in the cell transcribed from its DNA, before amplifying this co-DNA and sequencing it. The advantage of scRNA-seq is that mRNA is abundant in cells, making amplification and sequencing reliable with less noise introduced from over amplification and less chance of the amplification process missing key molecules. scRNA-seq, however is limited by the fact that it only analyzes the transcriptome - it only provides information on the exome and is influenced by transcription regulation, cell processes such as apoptosis and mRNA processing.

Single cell (or single nucleus) DNA sequencing, on the other hand, allows the whole genome of each cell to be amplified and sequenced directly. Since for each locus on a autosomal (diploid) chromosome only two molecules of DNA are present in each cell, however, the amplification process can lead to significant noise and erroneous artifacts in the data. Starting from only two (or one) molecules of DNA, the amplification process must go through many iterations (with more chance of error at each) and amplification errors at earlier iterations may overwhelm the amplified result. This can lead to various false-positive (FP) and false negative (FN) errors in the final sequencing data, with mutations observed from welltype DNA or conversely mutant DNA being sequenced as welltype.

Furthermore, in the case of autosomal diploid chromosomes, a further artifact known as allelic dropout (ADO) may be introduced in the SCS process. For a heterozygous locus on such a chromosome, only one of the pair of DNA molecules may be amplified leading to the locus being read as homozygous. If neither molecule succeeds in being amplified, there may be gaps the size of whole chromosomes in the resultant data.

Despite the FP and FN errors, as well as ADO artifacts and uneven coverage, the advantage of DNA SCS to potentially sequence the entire genome of single cells has proven a useful tool for analyzing genetic mutations at a per-cell basis. Algorithms analyzing such SCS data, however, must account for this distinct error profile and treat the data with appropriate flexibility.

1.4 Simplifications and Problem Scope

The biochemical processes underlying DNA mutations are incredibly complex, and modelling all the known (often context dependent) ways in which somatic DNA mutations may arise would be beyond the scope of any algorithm. For example, mutations may include insertions and deletions (indels) of various lengths; copy number variations (CNVs) where sequences of DNA are repeated a variable number of times; aneuploidy, where the normally diploid genome may become haploid or polyploid and even situations where the specific welltype nucleotides (ACGT) may make certain mutations more or less probable. With so much complexity in only the known patterns of mutation, modelling and analyzing all types of DNA mutation would be

beyond the scope of any bioinformatic algorithm. Some simplifications must therefore be introduced.

Following the example of state-of-the-art tools such as Monovar and SciPhi, only single nucleotide variations (SNVs, also called point mutations) are considered by SCarborSNV. For example indels are completely ignored, and CNVs are not inferred from read depth. Since aneuploidy is so prevalent in the context of cancers (TODO cite 90%), they are somewhat accounted for by SCarborSNV. SCarborSNV does account for changes in ploidy from diploid to haploid, which may result in a loss of heterozygosity (LOH), and models such events as a sudden switch from heterozygous to homozygous. Furthermore SCarborSNV considers all loci to be biallelic: only a reference and alternate allele are considered to be possible at each locus. A further area of research would be to infer and call all the various known types of DNA mutation, but it is outside the scope of this algorithm.

Finally, SCarborSNV assumes that the genome is composed of an infinite number of independent sites. This is to say that the prior probability of finding a reference or alternate allele at any locus is independent of surrounding loci, and furthermore at most one point mutation is possible at any given locus. The result is not only the biallelic assumption mentioned above, but also that each site may be analyzed in complete independence of its neighbours. As such, SCarborSNV (like Monovar and SciPhi) has linear complexity in the number of sites (denoted n). Although many mutations are in fact context dependent, and some loci are more or less vulnerable to mutation, the infinite sites assumption provides a great mathematical simplification and is therefore common among most SNV calling algorithms.

1.5 Phylogenetic Inference

For a set of m sequenced cells, there are 2^m possible phylogenetic trees that may relate them [TODO cite txt]. Furthermore if a point mutation has occurred at a locus, the mutation could have possibly occurred on any of the 2^m branches of the tree resulting in a total of 2^m possible structures to consider at any locus. A truly rigorous statistical analysis of the phylogenetic relationship between cells would have to compute the likelihood of the read data marginalized on all of these 2^m trees, which for any significant number of single cells is practically infeasible.

Since a rigorous search through the tree space is impractical, a heuristic search through a subspace of trees or an approximate phylogeny is required. Herein lies a major algorithmic challenge: using the knowledge of a shared ancestral relationship between cells to improve SNV calling accuracy while maintaining practical efficiency. The SciPhi algorithm uses an ingenious Markov chain Monte Carlo (MCMC) technique to heuristically search through a likely subspace of cell phylogenies and weight the final inference based on this subset of trees. The success of this method has proven the possibility of using phylogenetic inference to

improve SNV calling accuracy without incurring massive algorithmic complexity. The goal of SCarborSNV is to reduce this complexity further while still leveraging the prior information of cell kinship to improve calling accuracy over phylogeny-agnostic methods. In lieu of the MCMC algorithm employed by SciPhi, SCarborSNV uses an efficient neighbour-joining algorithm to infer information from approximate trees.

TODO: description of (probabilistic) jukes cantor and NJ pulled from txt.

The complexity of the core neighbour joining algorithm is $O(m^3)$ however only computing the pairwise distances involves scanning through the entire genome. Therefore the overall complexity of the neighbour joining algorithm is $O(nm^2 + m^3) = O(m^2(n + m))$, and since the size of n (possibly upwards of 3×10^9) dominates m (currently < 100 , possibly up to the order of 10^3 in future), for all practical purposes the neighbour joining in SCarborSNV is linear in n and quadratic in m . This potential for an asymptotic speedup is the core motivation for SCarborSNV, and may hopefully expand the possibility of single cell SNV calling to samples of thousands of cells.

2 Methods

The mathematical outline for calling SNVs is as follows. We begin by considering the total alternate allele count at any given locus, $\sigma \in [0, 2m]$, which represents the sum over all cells of the number of non-reference alleles at the locus. For a locus, we can compute the prior probability $P(\sigma)$ by considering various possible mutation histories and incorporating constants such as the prior probability of any mutation at a site (λ).

Next, we consider the likelihood of individual alternate allele count (g) on a per-cell basis: $P(d_{ij} \mid g)$. Using the prior $P(\sigma)$ and the cell likelihoods $P(d_{ij} \mid g)$, we use a dynamic programming algorithm to determine the posterior probability $P(\sigma \mid D_i)$ for each locus in $O(nm^2)$ time. At this stage we can exclude loci from further consideration for which $P(\sigma = 0 \mid D_i)$ is very high.

Having found the probability distribution for σ at each site using information from across all cells, we use this in a simple binomial model to gain locus-specific priors for individual cell genotypes. Using these priors and the cell genotype likelihoods we quickly find a posterior probability on individual cell genotypes, $P(g_{ij} \mid D_i)$ from a weighted sum over the σ distribution at each locus.

Using these posterior probability distributions of g for each cell at each locus, we define a new quantity $\overline{p_{ab}}$ that corresponds to the expected frequency with which alleles from cell a will differ from those from cell b . This expectation value $\overline{p_{ab}}$ can be used to define a genetic distance between any two cells d_{ab} inspired by the Jukes-Cantor distance. Computing all pairwise distances between cells can be completed in $O(nm^2)$.

An approximate phylogenetic tree of cells can be constructed using an algorithm known as neighbor joining.

TODO: rework once thought more about it. Inferring final cell genotypes from this phylogeny is achieved using a two-stage “upwards-downwards” algorithm for each locus. In the upwards stage, each branch of the tree is assigned a probability of a mutation occurring at that branch for this locus, using the posterior genotype probabilities of the cells on the leaves. Once the branches have been assigned marginal mutation probabilities, these probabilities can be propagated downwards from the root of the tree to return final phylogeny-aware genotype probabilities for all the cells in the leaves.

TODO: reestimate global parameters first, rebuild tree. Then maybe use EM to rebuild and recompute genotype probabilities??

2.1 Preprocessing and parallelism

Compute priors for σ and store somewhere where all processes can read. (shared memory, file)

Create unique identifiers for each cell. Each cell should be in own aligned BAM file.

Pile up aligned BAMs on loci.

- Use Python’s PySam pileup to iterate through columns (loci).
- Workers pick up batches of 1000? loci. Since sites assumed to be independent synchronicity is not important if locus positions and cellIDs are tracked.
- Workers process batch through to genotype calculation so not all are on I/O at once.
- Return locus objects with cells’ read and qual info at that locus.

Mark or discard indels and low/no coverage.

Impute welltype from germline data.(?)

- If germline VCF file provided, impute here
- Optionally use dbSNP as prior for germline vs somatic mutation

2.2 Cell genotype likelihoods

Calculate genotype likelihoods for each cell j at each locus i . We assume independence between sites.

Homozygous genotypes

- Let $g \in \{0, 1, 2\}$ be the unphased genotype of a locus designated by the number of non-reference alleles. For homozygous genotypes (that is, $g \in \{0, 2\}$) We generally assume reads to be independent:

$$P(D_{ij} | g) = \prod_{k=1}^n P(d_{ijk} | g) \quad (1)$$

Note $D_{ij} = (\vec{r}, \vec{e})$, where \vec{r} are the n reads at this nucleotide and this cell. \vec{e} are the associated probabilities of read error, derived from the phred quality scores.

- Marginalizing on sequencing error:

$$P(d_k | g) = P(r_k, se | g) + P(r_k, \neg se | g)$$

- Since errors can occur during amplification or sequencing, we model an "intermediate allele", denoted β that is amplified from the original nucleotide with some probability of error [1]. Trivially:

$$P(r_k, \neg se | g) = P(r_k | \neg se, g)(1 - e_k) = P(\beta_k = r_k | g)(1 - e_k)$$

We similarly see:

$$P(r_k, se | g) = P(r_k | se, g)e_k$$

Furthermore:

$$P(r_k | se, g) = P(r_k | \beta_k \neq r_k, se, g)P(\beta_k \neq r_k | se, g)$$

Assuming (?) an error in sequencing the intermediate allele could produce any of the other three alleles with equal probability we find $P(r_k | \beta_k \neq r_k, se, g) = 1/3$. Since the amplification of β is unaffected by sequencing $P(\beta_k \neq r_k | se, g) = P(\beta_k \neq r_k | g) = 1 - P(\beta_k = r_k | g)$. We therefore have:

$$P(r_k, se | g) = e_k \frac{1}{3} [1 - P(\beta_k = r_k | g)]$$

- Finally the likelihood $P(D_{ij} | g)$ for cell at a locus for a homozygous genotype is:

$$P(D_{ij} | g) = \prod_{k=1}^n \left[(1 - e_k)P(\beta_k = r_k | g) + e_k \frac{1}{3}(1 - P(\beta_k = r_k | g)) \right] \quad (2)$$

Heterozygous genotypes and allelic dropout

- For the heterozygous case, we must account for the possibility of allelic dropout (ADO) [1,2]. Therefore:

$$P(D_{ij} \mid g = 1) = P(D_{ij}, \text{ADO} \mid g = 1) + P(D_{ij}, \neg\text{ADO} \mid g = 1)$$

Letting P_{ADO} be the probability of an a dropout event, this expands to:

$$P(D_{ij} \mid g = 1) = P_{ADO}P(D_{ij} \mid \text{ADO}, g = 1) + (1 - P_{ADO})P(D_{ij} \mid \neg\text{ADO}, g = 1)$$

In the result of an allelic dropout from a heterozygous locus, only one allele will remain after the amplification process and hence the likelihood $P(D_{ij} \mid \text{ADO}, g = 1)$ will resemble the homozygous case. We assume allelic dropout can affect either allele with equal probability and hence:

$$P(D_{ij} \mid \text{ADO}, g = 1) = \frac{1}{2}P(D_{ij} \mid g = 0) + \frac{1}{2}P(D_{ij} \mid g = 2)$$

For the case without allelic dropout, the form of the likelihood is identical to the homozygous case:

$$P(D_{ij} \mid \neg\text{ADO}, g = 1) = \prod_{k=1}^n \left[(1 - e_k)P(\beta_k = r_k \mid g = 1) + e_k \frac{1}{3}(1 - P(\beta_k = r_k \mid g = 1)) \right]$$

2.3 Mutated site priors

We now focus on the prior probability of the total alternate allele count being σ at the locus under consideration: $P(\sum_j g_{ij} = \sigma) = P(\sigma)$. The majority of sites will not include a somatic SNV (sSNV); we say that any site has a prior probability λ of having a somatic SNV, which is set to 0.0001 by default [1,2].

$$P(\sigma) = P(\sigma \mid \text{sSNV})\lambda + P(\sigma \mid \neg\text{sSNV})(1 - \lambda) \quad (3)$$

Any given sample of single cells will only represent some subtree of a full cell phylogeny. As such, when considering the case where there is a sSNV at the locus we can further break down the prior into the case where the sSNV is ancestral to all sampled cells and the case where where the SNV occurs within the subtree rooted at the most recent common ancestor (MRCA) of the cells sampled. We denote the case where the mutation occurs within this subtree as SNV_T .

$$P(\sigma \mid \text{sSNV}) = P(\sigma \mid \text{SNV}_T)P(\text{SNV}_T \mid \text{sSNV}) + P(\sigma \mid \text{sSNV}, \neg\text{SNV}_T)(1 - P(\text{SNV}_T \mid \text{sSNV})) \quad (4)$$

Ploidy changes

It is well known that many tumor cells may exhibit aneuploidy or chromosomal abnormalities [5, 6]. For simplicity, we will disregard polyploidy and focus only on the case where loci become haploid. This sort of mutation can result in lost information regarding SNVs, as a loss of heterozygosity can lead to a locus being read as homozygous [2]. Note that this is an *in vivo* effect, distinct from allelic dropout which occurs *in vitro* during DNA amplification. We will model such occurrences as a sudden switch to homozygosity, as we cannot reliably distinguish diploid homozygosity from haploidy in the genomic SCS data, which already has significantly uneven coverage and depth [7]. Let H be the event that the locus under examination has become haploid, and H_T be the case that this mutation has occurred within subtree rooted at the MRCA of all sequenced cells.

$$P(\sigma \mid \text{SNV}_T) = P(\sigma \mid \text{SNV}_T, H)P(H) + P(\sigma \mid \text{SNV}_T, \neg H)(1 - P(H)) \quad (5)$$

We initially set the value of $P(H)$ to .09 (See Appendix A). In the simplest case, we consider the prior probability of an alternate allele count of σ given a mutation occurred within the subtree and the locus remained diploid across all sampled cells. Assuming infinite sites, in such a scenario mutations would only be heterozygous.

$$P(\sigma \mid \text{SNV}_T, \neg H) = \begin{cases} \frac{2m-1}{2(m-1)} T(m, \sigma) & 0 < \sigma < m \\ 0 & \text{else} \end{cases}$$

Where $T(m, \sigma)$ is the prior developed by Singer, Kuipers et al. that assumes a mutation may occur on any branch of the sampled subtree with equal probability.

$$T(a, b) = \frac{\binom{a}{b}^2}{(2b-1)\binom{2a}{2b}} \quad (6)$$

Now let us consider the case where both a sSNV and a haploid event have both occurred at a locus. Since the haploid mutation may have occurred in the subtree or ancestral to the subtree, we model these cases separately.

$$P(\sigma \mid \text{SNV}_T, H) = P(\sigma \mid \text{SNV}_T, H_T)P(H_T \mid H) + P(\sigma \mid \text{SNV}_T, H, \neg H_T)(1 - P(H_T \mid H))$$

SNV and loss of heterozygosity within the subtree

In the first scenario described by Equation 2.3 both a point mutation and a ploidy change have occurred within the sequenced subtree. This can be split into four further subcases (Figure 1).

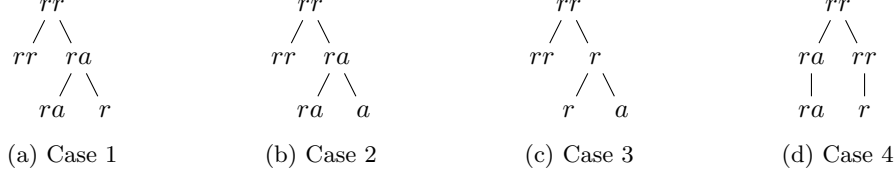


Figure 1: SNV and haploid event both within the subtree. (a) Point mutation happens before haploid event and mutated allele is dropped. (b) Point mutation happens before haploid event and reference allele is dropped. (c) Haploid event occurs before point mutation. Since haploid cells are modelled as becoming homozygous diploids, this case leads to only even values of alternate allele count. (d) In this case the point mutation and haploid event do not occur in the same lineage. We ignore this case as the haploid event does not affect the alternate allele count.

Considering the above three cases where a ploidy change in the subtree affects the locus alternate allele count, it is twice as likely that the point mutation should occur before the haploid event (cases 1 and 2), compared with the other temporal ordering (case 3). Therefore $P(\text{case 1 or 2}) = 2/3$ and $P(\text{case 3}) = 1/3$. This is because the cells before a haploid event, being diploid, have twice the chance of having a point mutation at a locus than the haploid descendants of such a mutation. We also assume the reference and alternate alleles have an equal chance of being dropped in a loss of heterozygosity.

$$P(\text{case 1}) = P(\text{case 2}) = P(\text{case 3}) = 1/3$$

Continuing to assume that both a point mutation and a haploid event have occurred within the sequenced subtree at the locus in question, we now have

$$P(\sigma \mid \text{SNV}_T, H_T) = \frac{1}{3} [P(\sigma \mid \text{case 1}) + P(\sigma \mid \text{case 2}) + P(\sigma \mid \text{case 3})]$$

To examine these probabilities we will use the function $T(a, b)$ described above, which given a subtree with a leaves gives the probability of a mutation affecting b of those leaves. For case 1, the loss of heterozygosity effectively deletes all alternate alleles from the cells sharing a lineage with the haploid event, and so

$$P(\sigma \mid \text{case 1}) = \frac{2m-1}{2(m-1)} \sum_{a-h=\sigma} T(m, a)T(a, h) \quad 1 \leq a < m, 1 \leq h \leq a$$

While we include one normalization constant, excluding the case of an ancestral point mutation, we do allow the case where a point mutation and a loss of heterozygosity happen on the same branch of the

phylogeny. Hence the support for σ in case 1 is $[0, m)$. Since we model a haploid event as a sudden switch to homozygosity, the observed allele count for case 2 is the number of cells affected by the heterozygous point mutation (a) added to the number of cells affected by the loss of heterozygosity (h).

$$P(\sigma \mid \text{case 2}) = \frac{2m-1}{2(m-1)} \sum_{a+h=\sigma} T(m, a)T(a, h) \quad 1 \leq a < m, 1 \leq h \leq a$$

For case 2, the support is $[2, 2m-2]$. In case 3, only even allele counts can be produced as all cells carrying the point mutation are haploid, which we model as being homozygous mutated. The possible values of σ are $1 \leq \sigma \leq 2m-2$.

$$P(\sigma \mid \text{case 3}) = \frac{2m-1}{2(m-1)} \sum_{2a=\sigma} T(m, h)T(h, a) \quad 1 \leq h < m, h \geq a$$

Haploid subtree

Referreing back to Equation (2.3), we must determine the prior probability of an alternate allele count σ at a locus given that a point mutation occured within the sequenced subtree and a haploid event occured ancestral to the subtree. This would lead to all sampled cells being haploid at this locus, therefore allowing only even allele counts.

$$P(\sigma \mid \text{SNV}_T, H, \neg H_T) = \begin{cases} \frac{2m-1}{2(m-1)} T(m, \frac{\sigma}{2}) & 2 \mid \sigma, 0 < \sigma < 2m \\ 0 & \text{else} \end{cases}$$

Clonal and subclonal mutations

We have so far considered the case where sSNVs have been subclonal: they may affect some of our sampled cells and not others. There is some probability however that a sSNV at a given locus may be due to a mutation in a cell ancestral to all sampled cells. The majority of these ancestral mutations will affect all tumour cells: so-called clonal, truncal or public mutations [4, 6, 8]. If the sample of single cells is small enough, however, it could be the case that a subclonal mutation is common to all cells sampled.

$$P(\text{ancestral} \mid \text{sSNV}) = P(\text{clonal}) + P(\text{ancestral} \mid \text{subclonal})(1 - P(\text{clonal})) \quad (7)$$

To find the probability of a subclonal mutation affecting all sampled cells, we assume tumour subclones follow a neutral evolutionary model such that subclonal mutant allele frequencies follow a power law distribution [8]. Using an IID model for tumour cell sampling, the probability that all m cells are from a subclone

with cellular frequency $2f$ (allelic frequency $= f$) is $(2f)^m$. Similar to Williams et al. we define a probability density function for the allelic frequency of subclonal mutations proportional to the inverse of the allelic frequency.

$$P(f) = k \left(\frac{1}{f} - 2 \right) \quad (8)$$

where k is a normalization constant. We define the support of $P(f)$ for subclones as $[10^{-8}, 0.5]$, as a frequency of 10^{-8} is on the order of affecting single cells and an allelic frequency of 0.5 corresponds to clonal mutations [9]. We find a value of k by integrating $P(f)$ over this support. If a subclonal mutation affects all sampled cells, then all these cells must be from the same subclone.

$$P(\text{ancestral} \mid \text{subclonal}) = \int_f P(\text{all in subclone} \mid f) P(f) df = \int_{10^{-8}}^{0.5} (2f)^m k \left(\frac{1}{f} - 2 \right) df \quad (9)$$

For large enough samples ($m > 30$) the probability that all cells were sampled from a single subclone (Equation 9) becomes negligible. Since it is only a function of m these values are pre-computed for efficiency. The empirically estimated probability that any given mutation is clonal is set at $P(\text{clonal}) = 0.51$ (see appendix A) [4, 6, 10]. Having developed the probability that any given somatic mutation is ancestral to all sampled cells, we have also found the probability that a mutation has occurred within the sampled subtree.

$$P(\text{SNV}_T \mid \text{sSNV}) = P(H_T \mid H) = 1 - P(\text{ancestral} \mid \text{sSNV})$$

Ancestral sSNVs

Referring Equation (4) we must also determine the prior for σ in the case that the sSNV is ancestral to all cells. Without LOH this would always result in $\sigma = m$, however we must again consider haploid events both within and ancestral to the sampled subtree.

$$P(\sigma \mid \text{sSNV}, \neg \text{SNV}_T) = P(\sigma \mid \text{sSNV}, \neg \text{SNV}_T, H)P(H) + P(\sigma \mid \text{sSNV}, \neg \text{SNV}_T, \neg H)(1 - P(H)) \quad (10)$$

If there is no haploid event, there will be no LOH and the ancestral sSNV will be heterozygous across all cells.

$$P(\sigma \mid \text{sSNV}, \neg \text{SNV}_T, \neg H) = \begin{cases} 1 & \sigma = m \\ 0 & \text{else} \end{cases}$$

In the case of a haploid event, we must consider the cases where the whole subtree is haploid and when the haploid event happens within the subtree. We also consider the alternate and reference alleles to be dropped with equal probability as above.

$$P(\sigma \mid \text{sSNV}, \neg\text{SNV}_T, H) = (1 - P(H_T \mid H))P(\sigma \mid \text{sSNV}, \neg\text{SNV}_T, H, \neg H_T) \\ + P(H_T \mid H)P(\sigma \mid H_T, \text{SNV}, \neg\text{SNV}_T)$$

If both the somatic SNV and the haploid event are ancestral to the sequenced subtree, the cells will either all be haploid reference or all be haploid alternate with equal probability. Since we model haploid cells as homozygous diploid this results in only alternate allele counts of 0 or $2m$.

$$P(\sigma \mid \text{sSNV}, \neg\text{SNV}_T, H, \neg H_T) = \begin{cases} \frac{1}{2} & \sigma = 0, 2m \\ 0 & \text{else} \end{cases}$$

If there is an sSNV ancestral to the sampled cells but a haploid event occurred within the sampled subtree we may have any alternate allele count from 1 to $2m - 1$ depending on where in the phylogeny heterozygosity was lost and which allele was dropped.

$$P(\sigma \mid \text{sSNV}, \neg\text{SNV}_T, H_T) = \begin{cases} \frac{1}{2}T(m, \sigma - m) & \sigma > m \\ \frac{1}{2}T(m, m - \sigma) & m > \sigma \end{cases}$$

2.4 Welltype site priors

We have so far considered the prior probability of alternate allele counts at a locus given that a somatic SNV has occurred at that locus. The majority of loci, however, will be unaffected by sSNVs, although may still contain germline point mutations. Referring back to Equation (3) we must consider the prior probabilities of σ for sites without sSNVs. Note, however, that such a site may still be affected by aneuploidy.

$$P(\sigma \mid \neg\text{sSNV}) = P(\sigma \mid \neg\text{sSNV}, H)P(H) + P(\sigma \mid \neg\text{sSNV}, \neg H)(1 - P(H)) \quad (11)$$

In the case where there is no sSNV and no aneuploidy, we simply assume Hardy-Weinberg equilibrium, with a germline mutation rate of μ . We set the value of μ relatively high at 0.1 to reduce false positive

errors.

$$P(\sigma \mid \neg \text{sSNV}, \neg H) = \begin{cases} \mu^2 & \sigma = 2m \\ 2\mu(1 - \mu) & \sigma = m \\ (1 - \mu)^2 & \sigma = 0 \\ 0 & \text{else} \end{cases}$$

Continuing to assume HWE for the germline genotype, aneuploidy will only affect the alternate allele count for a heterozygous germline genotype. Here again we assume either allele may be dropped with equal probability.

$$P(\sigma \mid \neg \text{sSNV}, H) = \begin{cases} \mu^2 + \mu(1 - \mu)(1 - P(H_T \mid H)) & \sigma = 2m \\ \mu(1 - \mu)P(H_T \mid H) \frac{2m-1}{2(m-1)} T(m, m - \sigma) & 0 < \sigma < m \\ 0 & \sigma = m \\ \mu(1 - \mu)P(H_T \mid H) \frac{2m-1}{2(m-1)} T(m, \sigma - m) & m < \sigma < 2m \\ (1 - \mu)^2 + \mu(1 - \mu)(1 - P(H_T \mid H)) & \sigma = 0 \end{cases}$$

2.5 Variant candidate site calling

To improve algorithmic efficiency we wish only to consider sites with a non-trivial posterior probability of containing a somatic mutation. Furthermore it has been shown that combining low coverage sequencing data across samples at a locus can decrease false positive rates [11]. We therefore must reject loci where the posterior probability of mutation is low. For a given locus i :

$$P(\text{SNV}_i \mid D_i) = 1 - P\left(\sum_{j=1}^m g_{ij} = 0 \mid D_i\right) = 1 - P(\sigma = 0 \mid D_i) \quad (12)$$

Using Bayes' formula:

$$P(\sigma \mid D_i) = \frac{P(D_i \mid \sigma)P(\sigma)}{\sum_{\sigma'=0}^{2m} [P(D_i \mid \sigma = \sigma')P(\sigma')]} \quad (13)$$

The value of $P(D_i \mid \sigma = 0)$ is simply the product of the cell likelihoods of homozygous reference calculated above. The priors $P(\sigma)$ are those determined by Equation (3). To compute the denominator, however, we must compute the likelihood for each alternate allele count across a locus. There are various permutations of cell genotypes that may give rise to an alternate allele count of σ , so this is not as simple as the special

case where $\sigma = 0$.

Let the phased genotypes of all m cells at a site be represented by $\vec{G} = (G_1, G_2, \dots, G_m)$ where $G_j \in [0, 1] \times [0, 1]$ is the phased genotype for cell j ($0 = \text{reference}$, $1 = \text{alternate}$). Furthermore let the unphased genotype vector be $\vec{g} = (g_1, g_2, \dots, g_m)$ be such that $g_j = \|G_j\|_1$. Our likelihood for σ can therefore be considered

$$P(D_i | \sigma_i) = \sum_{\vec{G}} P(D_i | \vec{G}) P(\vec{G} | \sigma_i) \quad (14)$$

We assume that all phased genotype vectors with a total alternate allele count of σ are equally probable. Since there are $\binom{2m}{\sigma}$ different phased genotype vectors with total alternate allele count σ , then for any such \vec{G} :

$$P(\vec{G} | \sigma) = \binom{2m}{\sigma}^{-1}$$

Since we do not consider phased sequencing data, we must reproduce Equation (14) in an unphased form. To begin, we see that the likelihood $P(D_i | \vec{G}) = P(D_i | \vec{g})$ if \vec{g} is the unphased vector that corresponds to \vec{G} , since our cell genotype likelihoods do not consider phasing. Note that there are $2^{\chi(\vec{g})}$ phased genotype vectors that correspond to any given unphased genotype vector \vec{g} , where $\chi(\vec{g})$ is the number of heterozygous cells in the vector. Using this multiplicity, we can now reproduce Equation (14) without reference to phasing.

$$P(D_i | \sigma_i) = \sum_{\{\vec{g}: \|\vec{g}\|=\sigma\}} \frac{2^{\chi(\vec{g})}}{\binom{2m}{\sigma}} P(D_i | \vec{g}) = \sum_{\{\vec{g}: \|\vec{g}\|=\sigma\}} \frac{2^{\chi(\vec{g})}}{\binom{2m}{\sigma}} \prod_{j=1}^m P(D_{ij} | g_j)$$

Let the function $\delta(\vec{g}, \sigma) = 1$ if $\|\vec{g}\| = \sigma$ otherwise it evaluates to 0. We can now write the above in a more suggestive form:

$$P(D_i | \sigma_i) = \binom{2m}{\sigma_i}^{-1} \sum_{g_1=0}^2 \sum_{g_2=0}^2 \cdots \sum_{g_m=0}^2 \delta((g_1, \dots, g_m), \sigma_i) \left[\prod_{j=1}^m \binom{2}{g_j} P(D_{ij} | g_j) \right] \quad (15)$$

As has been done previously, we can employ a dynamic programming approach to compute these likelihoods for σ from cell genotype likelihoods [1, 2, 11]. If we let $F(k, l)$ be the subproblem objective given by

$$F(k, l) = \begin{cases} \sum_{g_1=0}^2 \sum_{g_2=0}^2 \cdots \sum_{g_k=0}^2 \delta((g_1, \dots, g_k), l) \left[\prod_{j=1}^k \binom{2}{g_j} P(D_{ij} | g_j) \right] & 0 \leq l \leq 2k \\ 0 & \text{else} \end{cases} \quad (16)$$

We can consider creating a genotype vector of length k from a vector of length $k - 1$ by adding one new cell

with an alternate allele count of 0, 1 or 2. Hence our recurrence relation can be given by

$$F(k, l) = F(k-1, l)P(D_{ik} | g_k = 0) + 2F(k-1, l-1)P(D_{ik} | g_k = 1) + F(k-1, l-2)P(D_{ik} | g_k = 2) \quad (17)$$

Note that two possible phased genotypes correspond to the heterozygous case, hence the factor of 2 in the second term. The base case where $k = 1$ corresponds to a single cell

$$F(1, 0) = P(D_{i1} | g_1 = 0), \quad F(1, 1) = 2P(D_{i1} | g_1 = 1), \quad F(1, 2) = P(D_{i1} | g_1 = 2)$$

The values for $F(k, l)$ are memoized in an array and the likelihood given in Equation 15 can be given by

$$P(D_i | \sigma_i) = \frac{F(m, \sigma_i)}{\binom{2m}{\sigma_i}} \quad (18)$$

In this way we can determine the likelihood of all $0 \leq \sigma \leq 2m$ which when the priors $P(\sigma)$ compose the sum in Equation (13).

Sites which have a posterior probability of being variant greater than 0.5(???) will be called as variant candidates.

2.6 Building a cell phylogeny

The most accurate phylogenetic structure of the sampled tumour cells could be found by searching through the entire tree space and finding a tree that maximizes likelihood or posterior probability. If s candidate sites are called in the previous step there are $(2m-3)!!(2m-1)^s$ trees in the search space making this approach infeasible, leading a previous phylogeny aware approach to adopt a more efficient Markov chain Monte Carlo (MCMC) algorithm [2]. This more efficient approach results in an overall asymptotic complexity of $O(nm^3 \log(m))$ [2]. The Monovar algorithm has an overall asymptotic complexity of $O(nm^3)$ [1].

We use a simple neighbour-joining algorithm to infer a cell phylogeny based on the sites called as variant candidates. This approach has an asymptotic complexity of $O(nm^2 + m^3)$, and so especially for data sets with many cells we expect it will yield results faster even than Monovar. Monovar determines cell genotype posteriors by simultaneously considering the cell in question and the probability of all other cells having allele count $\sigma - g$ and pooling data across all cells. First, we calculate alternate allele priors for a site using read data from all the cells, where $P(\sigma) = P(\sigma | D_i)$ can be determined using Bayes' formula using the memoized values computed for Equation 13. After this step, we make the simplification that $P(D_i | g_{ij}) = P(d_{ij} | g_{ij})$.

Therefore using Bayes' formula and marginalizing on σ :

$$P(g_{ij} | D_i) = P(g_{ij} | d_{ij}) = \sum_{\sigma=0}^{2m} \left[P(\sigma) \frac{P(d_{ij} | g_{ij})P(g_{ij} | \sigma)}{\sum_g P(d_{ij} | g)P(g | \sigma)} \right]$$

where $P(d_{ij} | g)$ are simple cell genotype likelihoods. For the conditional prior on cell likelihoods, we use a simple binomial approximation that can be pre computed for all sites

$$P(g | \sigma) = \binom{2}{g} \left(\frac{\sigma}{2m} \right)^g \left(1 - \frac{\sigma}{2m} \right)^{2-g}$$

Next we define a pairwise value \bar{p} , the expected frequency with which nucleotides differ between two cells a and b :

$$\bar{p} = \frac{1}{2n} \sum_{i=1}^n \left[\sum_{|g_{ia}-g_{ib}|=2} 2P(g_{ia})P(g_{ib}) + \sum_{|g_{ia}-g_{ib}|=1} P(g_{ia})P(g_{ib}) \right]$$

Note we assume that if two cells have the same alternate allele count at a locus they have the same phased genotype at that locus. We then compute a distance inspired by the Jukes-Cantor distance:

$$d = -\frac{3}{4} \log \left(1 - \frac{4}{3} \bar{p} \right) \quad (19)$$

Included with the biological cells is a false cell with reference genotype which is included as an outgroup to root the tree. After computing d for all pairs of cells, we implement a simple neighbor-joining algorithm based thereon [12].

2.7 Genotyping single cells

Assuming the tree created above is accurate, we now seek to infer genotypes from this phylogeny so as to overcome errors and noise associated with low coverage SCS data. We first determine weights for attaching point mutation and different types of LOH events to different edges of the tree, and then use these weights to determine genotype probabilities for each cell.

2.7.1 SNV weights

To begin with, we compute the probabilities of all descendants of each node having the same genotype: $\pi_0(e)$, $\pi_1(e)$ and $\pi_2(e)$ are the probabilities that all descendants of e are homozygous reference, heterozygous

and homozygous alternate respectively. These values are taken to be

$$\pi_g(e) = \prod_{\{j:c_j \succ e\}} P(g_j = g)$$

where $c_j \succ e$ indicates that the j^{th} cell is below e in T , and $P(g_j = g)$ are the posterior probabilities calculated in Equation (13). We also compute one more values, $\pi_\mu(e)$, defined as the probability that all descendents of e have genotype 1 or 2. These four values can be computed recursively in $O(m)$ time by multiplying the corresponding values from the two branches directly beneath each branch. In the case of a point mutation but no loss of heterozygosity, the weight given to attaching a point mutation at edge e is given by:

$$W(S_e) = d_e \pi_\mu(e) / \pi_0(e) \quad (20)$$

The probability that a mutation occurred at edge e is given by (see appendix ??):

$$P(S_e | T, D) = (1 - P(\sigma = 0)) \frac{W(S_e)}{\sum_{e' \in E} W(S_{e'})}$$

Weights For Loss of Heterozygosity

Referring to Figure ?? above these weights are calculated for cases 1, 2 and 3 in the following way:

$$W^{(1)}(S_{e_1}, L_{e_2}) = \frac{\pi_0(e_2) \pi_1(e_1)}{\pi_1(e_2) \pi_0(e_1)} d_{e_1} P(L_{e_2})$$

$$W^{(2)}(S_{e_1}, L_{e_2}) = \frac{\pi_2(e_2) \pi_1(e_1)}{\pi_1(e_2) \pi_0(e_1)} d_{e_1} P(L_{e_2})$$

and

$$W^{(3)}(S_e) = \frac{\pi_2(e)}{\pi_0(e)} d_e$$

If a haploid event should occur at the locus under examination, the prior probability that it should occur on edge e is given to be:

$$P(L_e) = \frac{d_e}{\sum_{e' \in E - E_l} d_{e'}}$$

where E_l is the set of all edges directly above leaf nodes. It is assumed that allelic dropout due to amplification is much more frequent than ploidy changes that affect only a single cell, and thus the prior probability of the latter is set to 0. For cases 1 and 2 we define

$$W'^{(1)}(L_e) = \frac{\sum_{e' \preceq e} W^{(1)}(S_{e'}, L_e)}{\sum_{e' \preceq e} W(S_{e'})}$$

where the values in the denominator come from Equation (20). Similarly

$$W'^{(2)}(L_e) = \frac{\sum_{e' \preceq_e} W^{(2)}(S_{e'}, L_e)}{\sum_{e' \preceq_e} W(S_{e'})}$$

Finally, assuming an SNV has already occurred at the given locus, the probability of a case 1 haploid event happening at any edge e is given by (see appendix ??):

$$P(L_e^{(1)}) = P(L_e^{(1)} | S_{\preceq_e}) = \frac{P(LOH)}{3} \frac{W'^{(1)}(L_e)}{\sum_{e' \in E-E_l} W'^{(1)}(L_{e'})}$$

and under the same assumption, the conditional probability for case 2:

$$P(L_e^{(2)}) = P(L_e^{(2)} | S_{\preceq_e}) = \frac{P(LOH)}{3} \frac{W'^{(2)}(L_e)}{\sum_{e' \in E-E_l} W'^{(2)}(L_{e'})}$$

The probability for case 3 is given by

$$P(L_e^{(3)}) = P(L_e^{(3)} | S_{\preceq_e}) = \frac{P(LOH)}{3} \frac{W^{(3)}(L_e)}{\sum_{e' \in E-E_l} W^{(3)}(L_{e'})}$$

The prior probability $P(LOH)$ of a loss of heterozygosity occurring at the site given that a SNV has occurred is given to be

$$P(LOH) = P(LOH_i | SNV_i) = \frac{3}{2} \cdot \frac{1 - \prod_j [P(g_{ij} = 0) + P(g_{ij} = 1)]}{1 - P(\sigma_i = 0)}$$

Genotyping Cells

With all of these values computed for each of the edges in the tree, we can use a dynamic programming algorithm to genotype the cells. We complete a depth first traversal of the tree keeping track of genotype probabilities at every node. We also keep track of the probability $P(L_{\preceq_e}^{(3)})$ that a silent case 3 haploid event has occurred ancestral to each node. The initial conditions for the root node are the genotype probabilities $P(g = 0) = 1$ and $P(g = 1) = P(g = 2)$ and $P(L_{\preceq_\rho}^{(3)}) = 0$. Let n_1 be the direct ancestor of n_2 such that n_1 and n_2 are joined by e and have genotypes g_1 and g_2 respectively. Therefore we define the relations:

$$P(L_{\preceq_{n_2}}^{(3)}) = P(L_{\preceq_{n_1}}^{(3)}) + P(L_e^{(3)})$$

$$\begin{aligned} P(g_2 = 0) = & P(g_1 = 0) \left[(1 - P(S_e)) + P(S_e)P(L_e^{(1)}) \right] \\ & + P(g_1 = 1) \left[P(L_e^{(1)}) \right] \end{aligned}$$

$$P(g_2 = 1) = P(g_1 = 0) \left[P(S_e)(1 - P(L_e^{(1)}) - P(L_e^{(2)})) \right] \\ + P(g_1 = 1) \left[1 - P(L_e^{(1)}) - P(L_e^{(2)}) \right]$$

$$P(g_2 = 2) = P(g_1 = 0) \left[P(S_e)(P(L_{\leq n_2}^{(3)}) + P(L_e^{(2)})) \right] \\ + P(g_1 = 1) \left[P(L_e^{(2)}) \right] \\ + P(g_1 = 2)$$

When the tree traversal reaches the leaf nodes, these are taken to be the cell genotype probabilities. The cell will be genotyped in accordance with the highest probability.

2.8 Additional computational methods

Stirlings approximation for $T(a, b)$. Pre computation /memoization of priors where possible. Log space.

3 Results

Include complexity analysis

4 Discussion

Two methods for leaves affected: power law and uniform branches. Uniform branches (sciphi) used for sampled vs power law for considering whole tumour. Cannot use power law for sample? Why? Why not?

Overall question: is $O(nm^3)$ good enough at all stages? (No!) asymptotically only beats sci ϕ by $\log(m)$...

Ofc NJ is $O(m^3)$ but not nm^3 since you build a single tree from info on all sites! So my alg could be bounded by $O(nm^2)$?? Computing distances in $O(nm^2)$... Could have an $O(nm^2)$ algorithm!

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A Clonal mutations and aneuploidy

Grid search, geometric ?? Do we do this or use empirical values?

B Initial Parameter tuning

Grid search, geometric ?? Do we do this or use empirical values?

C Tree genotype posteriors

Instead of d_e , we should likely use $1 - \exp(-\frac{4}{3}d_e)$ as it should be proportional to the frequency with which sites between two cells differ.

C.0.1 SNV weights

To begin with, we compute the probabilities of all descendants of each node having the same genotype: $\pi_0(e)$, $\pi_1(e)$ and $\pi_2(e)$ are the probabilities that all descendants of e are homozygous reference, heterozygous and homozygous alternate respectively. These values are taken to be

$$\pi_g(e) = \prod_{\{j:c_j \succ e\}} P(g_j = g)$$

where $c_j \succ e$ indicates that the j^{th} cell is below e in T , and $P(g_j = g)$ are the posterior probabilities calculated in Equation (13). We also compute one more values, $\pi_\mu(e)$, defined as the probability that all descendants of e have genotype 1 or 2. These four values can be computed recursively in $O(m)$ time by multiplying the corresponding values from the two branches directly beneath each branch. In the case of a point mutation but no loss of heterozygosity, the weight given to attaching a point mutation at edge e is given by:

$$W(S_e) = \frac{\pi_\mu(e) [\pi_0(\rho)/\pi_0(e)] P(S_e)}{\sum_{e' \in E} \pi_\mu(e') [\pi_0(\rho)/\pi_0(e)] P(S'_e)} = \frac{d_e \pi_\mu(e)/\pi_0(e)}{\sum_{e' \in E} d_{e'} \pi_\mu(e')/\pi_0(e')} \quad (21)$$

where ρ represents the root edge and hence $\pi_0(\rho)/\pi_0(e)$ is a product of probabilities over only those cells that do not descend from e . The prior probability of an edge containing a mutation is taken to be the normalized edge length:

$$P(S_e) = \frac{d_e}{\sum_{e'} d_{e'}}$$

Weights For Loss of Heterozygosity

We try find conditional probability of each type of LOH **Assuming** mutation above Idea: only cases 1 and 2 are complex. Case 3 can be modeled as a haploid full tree and case 4 is ignored. For cases 1 and 2 we can then work out conditional weights assuming mutation above the attachment points.

For a loss of heterozygosity events, we calculate weights in a similar way. Referring to Figure ?? above these weights are calculated for cases 1, 2 and 3 in the following way:

$$W^{(1)}(S_{e_1}, L_{e_2}) = \frac{\pi_0(e_2) [\pi_1(e_1)/\pi_1(e_2)] [\pi_0(\rho)/\pi_0(e_1)] P(S_{e_1})P(L_{e_2})}{\sum_{e'_1 \in E-E_l} \sum_{e'_2 \succeq e'_1} \pi_0(e'_2) [\pi_1(e'_1)/\pi_1(e'_2)] [\pi_0(\rho)/\pi_0(e'_1)] P(S_{e'_1})P(L_{e'_2})}$$

Are we looking to assume mutation and LOH type? No, these assume a specific configuration. Surely to get assumption of mutation must sum over all $e_1 \preceq e_2$. There are $O(m)$ edges so computing $W^{(1)}$ s takes $O(m^2)$ must then sum all with same e_2 , $O(m)$ Ws for each val of e_2 , $O(m)$ vals of e_2 so as long as algorithm done thoughtfully can get $O(m^2)$. To get conditional probs from these should normalize then *1/3

$$W^{(2)}(S_{e_1}, L_{e_2}) = \frac{\pi_2(e_2) [\pi_1(e_1)/\pi_1(e_2)] [\pi_0(\rho)/\pi_0(e_1)] P(S_{e_1})P(L_{e_2})}{\sum_{e'_1 \in E-E_l} \sum_{e'_2 \succeq e'_1} \pi_2(e'_2) [\pi_1(e'_1)/\pi_1(e'_2)] [\pi_0(\rho)/\pi_0(e'_1)] P(S_{e'_1})P(L_{e'_2})}$$

and

$$W^{(3)}(S_e) = \frac{\pi_2(e) [\pi_0(\rho)/\pi_2(e)] P(S_e)}{\sum_{e' \in E-E_l} \pi_2(e') [\pi_0(\rho)/\pi_2(e')] P(S'_e)}$$