A computational framework to infer the order of accumulating mutations in individual tumors

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Abstract

Phylogenetic methods are routinely used to quantify intra tumor heterogeneity (ITH) from multi-sample sequencing of individual tumors. These methods can deconvolve clonal or mutational structures, but sometimes require several complex technical assumptions. Here, we present a simple computational framework (Temporal oRder of Individual Tumors, TRaIT) to infer the qualitative ordering of mutations that accumulate during tumor growth from single-cell and multi-region data. TRaIT provides several off-the-shelf algorithms that can model confounding factors, tumors with multiple cells of origin and a generalized form of parallel (convergent) evolution. Our methods efficiently deal with technical errors in the data and have state-of-the-art performance, lower computational cost and better scalability than tools for phylogenetic inference. We show with real breast and colorectal cancer data that the joint application of TRaIT and complementary phylogenetic methods allows to better quantify the extent of ITH, and generate novel experimental hypotheses.

Background

The vast and increasing number of sequenced cancer genomes has revealed that most tumors are characterized by high levels of *intra-tumor heterogeneity* (ITH), due to the simultaneous coexistence of various (sub)clones with different mutational profiles [1–3]. Each subclone evolves by the progressive accumulation of distinct (epi)genomic alterations that confer specific functional advantages to its cells [4–6]. This leads to a wide subclonal variability in terms of growth rate, metastatic potential, ability of evading the immune system – often resumed as *phenotypic hallmarks* [7] – and, above all, therapeutic resistance [8–11].

Clones compete for limite resources (e.g., nutrients, oxygen, etc.) in alternated phases of Darwinian selection [12], neutral evolution [13] and punctuated

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dynamics [14]. This complex dynamics is reflected in the intricate spatial distribution of malignant cells in neoplastic lesions and metastases [15]. It is still unclear how (epi)genetic alterations interact to produce such hallmarks [16–18], and whether any order is favoured in the process of accumulation, since many alterations are often observed either as early or late events in the same tumor (sub)type [19–21].

Despite the huge efforts aimed at characterizing ITH, the inference of the (sub)clonal evolutionary history of tumors is still an open challenge, which might have dramatic clinical implications. To this end, in the last years a broad number of computational approaches have been developed to process distinct types of sequencing data, with different goals and properties (see [22, 23] for recent reviews).

Bulk sequencing of *single biopsies* (e.g., the TCGA data [24]) captures a mixture of signals from the various subpopulations of the tumor. These data were originally used to reconstruct *progression models* that recapitulate *heterogenity among patients*, rather then within a single tumor [25–31]. Subsequently, these data were also used to deconvolve ITH from *allelic frequencies*, via *phylogenetic* techniques [32–38].

Nowadays, it is acknowledged that single biopsies are poor predictors of actual ITH, and an increasing number of studies is relying on bulk sequencing from multiple spatially separated tumor regions – dubbed *multiple-biopsy/ region* [39–43]. Many of the aforementioned techniques can, in fact, process multiple samples from the same tumor. Despite the intrinsic limitations of bulk sequencing, these techniques have allowed to draw a preliminary quantitative picture of ITH, with high levels of ITH detected across several cancer types.

In principle, the best data to quantify ITH is produced by *single-cell DNA sequencing* (SCS) of individual cells, rather than mixtures [44]. Unfortunately, several challenges in cell isolation and genome amplification from single cells hinder SCS, and data harbour high levels of *allelic dropouts*, *missing data* and *non-uniform coverage* [45]. According to different studies, allelic dropout rates vary from 10% to 50% [46–50], and remain relevant despite the constant improvement of variant callers [51]. False positives are significantly less frequent, yet sometimes comparable to the number of true somatic mutations [46–50]. Also *doublet* errors, in which single sequencing libraries are accidentally generated from multiple cells, can affect the data by returning non existing mixtures of cell lineages [52]. Finally, the number of missing data can be dramatically large, sometimes exceeding 50% of the sequenced genotypes [47, 53].

This state of affairs prevents straightforward applications of *perfect phylogeny* algorithms to SCS data [54], and a growing number of *cancer-specific probabilistic approaches* to infer distinct types of tumor phylogenies from SCS data have being proposed [52, 55].

A first category of approaches estimates *the ordering of accumulating mutations and displays it as mutational trees* [56,57], yet without deconvolving clones' signatures. These models provide *qualitative temporal orderings*, that is statements like "mutation x is temporally precedent mutation y", with a measure of statistical confidence (e.g., p-values or posterior estimates). In this group, the first maximum-likelihood technique tailored to SCS data was proposed in [56]. At the time of this writing, a more efficient approach is implemented in SCITE, which couples a Markov Chain Monte Carlo sampling scheme to a model of noise to deal with imperfect data, and infers a full posterior over mutational

trees [57].

A second category of approaches *deconvolve the temporal and evolutionary relations among clones*, yet without explicitly ordering the mutations within clones [58–60]. BitPhylogeny, for example, employs a non-parametric Bayesian approach to infer such trees, yet displaying limited efficiency with the few samples available by SCS [58]. Single Cell Genotyper uses a mixture model and mean-field variation inference to identify clonal genotypes in presence of missing values and biased allelic counts [60]. A well performing approach is implemented in OncoNEM, which uses a heuristic search to reconstruct a condensed tree of the evolutionary relationships among subclones [59].

The cancer-specific techniques that we mentioned, as well as the standard approaches (e.g., *maximum parsimony*), ground their roots in phylogenetic theory. They are effective, but in general require specific assumptions concerning sequence substitution, alleles fixation, branching lengths estimation and noise deconvolution. Complex assumptions might lead to predictions that are not evidently supported by data, or might require disambiguation heuristics to select, *post hoc*, a unique output model. For instance, this happens when one needs to select, among a set of equivalent-scoring maximum parsimony inferences, a unique phylogenetic tree (see the case studies [39, 40]).

We here propose TRaIT – Temporal oRder of Individual Tumors –, a new simple computational framework to *infer the order of accumulating mutations, copy-numbers, etc. from SCS or multi-region bulk data.*

TRaIT employs few assumptions, and its models can be easily interpreted: if a model contains edge $x \to y$, then x^+ mutants are ancestral to y^+ ones, and y^+ mutants are *statistically associated* to x^+. These conditions describe the *underlying clock* among x and y, and are estimated by simple inequalities from data. These models display all input mutations in the final model, as mutational trees or other phylogenies do. Thus one should be warned that x and y could be *passenger events* observed by hijacking. To filter them out x and y, however, one would arguably need more complex tools and data combined with *causal* approaches [29–31].

In this simple temporal structure has several computational advantages, which we exploit to implement a suite of algorithms that capture different aspects of tumor evolution (Figure 1). TRaIT can process datasets where mutations unrelated to the progression are wrongly annotated (i.e., *confounding factors*). Within our computational framework, we will be informed of their poor statistical association. Also, TRaIT can detect *multiple independent progressions within the same dataset*, capturing tumor initiation by *multiple cells of origin* [61]. Besides, the framework can deal with *convergence of distinct evolutionary trajectories*, i.e., $x \to y$ and $z \to y$ in the same model. This last case extends parallel evolution towards the same gene (i.e., $x \to y$ and $z \to w$, where y and w are distinct mutations in the same gene) [39].

Tests of TRaIT on simulated data suggest state-of-the-art accuracy, significantly lower complexity and higher scalability with respect to methods specially tailored to infer mutational trees from SCS or multi-region data. Besides, our methods present potentially broader applications than tree-constrained phylogenies, as they can infer more complex temporal structures. By combining all the analyses available via TRaIT, and by coupling them with phylogenetic tools that deconvolve tumor clonal signatures, we believe that more comprehensive

pictures of tumor evolution and ITH could be drawn.

Results

TRaIT includes 4 optimal polynomial-time algorithms (Figure 2) that process a binary matrix D with n columns and m rows [57]. D stores n variables (mutations, CNAs, etc.) detected across m samples (single cells or multi-region samples). If an entry in D is 1, then the associated variable is detected in the sample. Missing data in SCS are handled by a standard EM procedure with multiple imputations [62]. A priori estimates of false positives/ negatives rates $\epsilon_+, \epsilon_- \geq 0$ in D can be provided to each algorithm. All the algorithms are implemented in R, in the TRONCO tool for Translational Oncology [63, 64].

At their core, TRaIT's algorithms exploit a bootstrap procedure to compute 2 p-values per edge – one for temporal direction, one for association's strength, according to Suppes' theory of *probabilistic causation* [65] (see Methods). Two algorithms infer *mutational trees* (Edm, Gbw) and two *direct acyclic mutational graphs* (DAGs) (ChL, PRIM) (for an extended definition of the algorithms please refer to the Methods Section and the Supplementary Material). All algorithms can return a model with separate components, suggesting that data lacks statistically significant associations, *or* harbours multiple progressions.

For these reasons, TRaIT can be used (Figure 1):

(i) when all D's variables are actually involved in the progression (i.e., all the events are *drivers*);

(ii) when some of D's variables are *confounding factors* (i.e., miscalled mutations);

(iii) when D contains multiple independent progressions (i.e., *multiple cells of tumor origin*);

(iv) when distinct evolutionary trajectories *converge* to the same variable (i.e., certain drivers are shared by distinct (sub)clonal histories).

Edm and Gbw infer models that may account for phenomena $(i, \ldots, iii)$, whereas only ChL and PRIM can explicitly account for case (iv). One can choose which TRaIT's algorithm to use according to, e.g., research goals or prior knowledge on the evolutionary process. A rule of thumb might be the application of all TRaIT's algorithms, followed by a comparative analysis of their output models – as we show in the case studies. The creation of a *consensus* model could be also effective to this end. In what follows we present results from simulations of different experimental conditions and data; these tests allowed to assess the performance of the algorithms in the four scenarios (see Simulations), thus providing some indications for an appropriate algorithmic choice according to the specific case.

Simulations

We assessed the performance of TRaIT's algorithms with simulated single cell and multi-region data.

In particular, we generated multiple batches of independent synthetic datasets from random phylogenies (generative models), with $5 \leq n \leq 20$ nodes and different levels of topological complexity (Figure 1D). SCS datasets with $10 \leq m \leq 100$ cells and multi-region datasets with $5 \leq m \leq 50$ regions (accounting for sampling bias) were created. To test the robustness against imperfect data, false positives, false negatives (highly asymmetric for SCS) and/or missing data were introduced in the true genotypes, consistently with previous studies [57]. Multiple configurations of parameters were scanned, and we measured the ability to infer true edges (*sensitivity*), and discriminate false ones (*specificity*); further details on data generation are available as Supplementary Material.

We compared our methods to SCITE, the state-of-the-art for phylogenetic inference of *mutational trees* from SCS data [57]. In the test, we also included previously developed approaches to causal inference from single-sample data (CAPRESE [29] and CAPRI [30]).

Full results are in Supplementary Figures 3 and 5–15. Here we show four simulations in Figure 3; these settings are consistent with the results across all tests. Figure 3 displays the results for TRaIT and SCITE[1] in canonical settings of noise and sample size, for case (*i*) (SCS and multi-region data), for case (*ii*) (multi-region data), and for case (*iii*) (SCS data).

All the techniques achieve high sensitivity and specificity scores from SCS generated by phylogenies with drivers only – Edm and Gbw highlighting the best results (medians approx. 0.8 and 1). When we sampled multi-region data from the same topology, performances worsened for all methods likely due to the smaller sample size and the mixed bulk signal. The introduction of confounding factors (2 out of $n = 13$ variables), does not to impact the performance significantly, and all algorithms mostly discriminate the true generative model. Finally, the inference of tumors initiating from multiple cells proves to be a harder task, as sensitivity decreases and the performance of all methods are similar. Notice that SCITE, in all tests, achieves the lowest specificity; this might point at a mild-tendency to overfitting, probably due to the combination of its search scheme and noise-learning model (see also Discussions).

Some general conclusions can be drawn from the whole set of tests that we carried out. As expected, performances improve with lower noise and larger datasets. In particular, with SCS data Gbw, Edm and SCITE seem the best algorithms; they generally achieve very similar sensitivity, even though the latter presents (on average) lower specificity. For SCS data, all the tested algorithms seem very efficient up to 20/30% of missing data, with SCITE showing a slightly greater robustness (Supplementary Figure 11).

Results on multi-region data display similar trends, with Gbw and Edm showing the overall best performance. In this case, however, SCITE is less effective in retrieving both the true and the false relations, especially with small datasets and/or low noise levels.

Interestingly, by systematically analyzing the impact of a variation of the input ϵ_+ and ϵ_- with respect to the true noise values, we discovered that the performance is rather stable for all algorithms (in Figure 3 we show Gbw algorithm). For this reason, we did not implement sophisticate noise-learning strategies in TRaIT.

[1] SCITE can return a posterior with many equivalent-scoring mutational trees; in those cases, to compute its error, we selected the first of those models.

Finally, a computation time assessment allowed to record a 3× speedup of all the algorithms included in TRaIT with respect to SCITE, on standard CPUs (Supplementary Table 10).

SCS data: Triple-Negative Breast Cancer

We applied TRaIT to a SCS dataset of Triple-Negative Breast Cancer (patient "TNBC" in [50]). The input data consists of single-nucleus exome sequencing of 32 cells (8 *aneuploid* (A) cells, 8 *hypodiploid* (H) cells and 16 normal cells (N)).

In [50], with a bulk sequencing control, mutations detected both in the bulk and in the majority of the cancer cells were annotated as *clonal*, whereas those undetected in the bulk as *subclonal*. The authors then manually curated a qualitative phylogenetic tree (Figure 4). We here run TRaIT with the mutational profiles of each single cell describing the presence/absence of *nonsynonymous point mutations* of the 22 genes selected in [50]. The rate of missing values in this dataset is very low (around 1%). Moreover, as suggested in [50], we provided to TRaIT a value of 9.73×10^{-2} for allelic dropout rate and 1.24×10^{-6} for false positive rate.

For these data, all TRaIT's algorithms return trees (Supplementary Figures 16-17); here, we discuss Edm's one since that algorithm achieved the best performance in the simulations with drivers and confounding factors. To improve the analysis, from the same dataset we also deconvolve the clonal signatures with OncoNEM, and compare the predictions (Figure 4).

TRaIT allows to characterize the qualitative phylogeny provided in [50] by identifying the gradual accumulation of point mutations, expectedly due to defects in DNA repair or replication machineries, both in the clonal and subclonal histories of the tumor.

On the one hand, Edm's model displays high-confidence branched evolution consistent with subclone A_1 (PPP2R1A, SYNE2 and AURKA mutations), A_2 (ECM2, CHRM5 and TGFB2 mutations), and H (NRRK1, AFF4, ECM1, CBX4 mutations) [50].

On the other hand, TRaIT provides a notably higher resolution in the description of the mutations annotated as clonal in [50], e.g., PTEN, TBX3 and NOTCH2, are suggested to trigger tumor initiation. These results are also consistent with the presence of different molecular clocks operating at different stages of tumour growth described in [50]. TRaIT allows to formulate new hypotheses about undetected subclones, possibly characterized by private mutations in AKAP9, or in JAK1, SETBP1 and CDH6, which however would require further experimental confirmations.

OncoNEM predicts the presence of 10 clones, their lineages and temporal relations, thus refining the qualitative analysis of [50]. Remarkably, such results are mostly consistent with ours, as the mutational ordering predicted by OncoNEM (obtained by estimating the assignment of mutations to clones, as suggested in [59]) largely overlaps with that inferred via TRaIT. This is particularly evident for early events, and for most of the late subclonal ones; exception made for subclone H, which is not detected by OncoNEM. As mutations in ARAF, AKAP9, NOTCH3 and JAK1 have the same marginal probability, their temporal ordering can not univocally determined from these data. TRaIT, in fact, provides a p-value $p > 0.05$ for the direction of those edges, suggesting that any permutation of their ordering would be possible. For this reason, unless more

sequenced cells were available, we can not univocally match the clonal signatures obtained with OncoNEM and the temporal orderings identified by TRaIT for these temporally-intermediate events.

This result proves that the concerted use of techniques for the inference of mutational ordering, together with clonal deconvolution approaches, can provide a picture of tumor evolution and ITH at an unprecedented resolution and accuracy.

Multiple-biopsy data: MSI-high Colorectal Cancer

We applied TRaIT to a moderately-differentiated MSI-High colon cancer characterized by a primary tumor and a right hepatic lobe metastasis, with no prior treatments (patient "P3" in [66]). For this patient, targeted DNA resequencing of three regions of the primary tumor (P3-1, P3-2, and P3-3) and of two metastatic regions (L-1 and L-2), allowed to identify 47 *nonsynonymous point mutations* and 11 *indels* [66].

To process this dataset with TRaIT, we first grouped the mutations with the same signature across the five regions, hence obtaining: (*a*) a clonal group, including the 34 mutations detected in all the samples, (*b*) a subclonal group, including the 3 mutations detected only in the L regions, and (*c*) 8 mutations with different mutational profiles. Our methods will not resolve their ordering since their signals are statistically undistinguishable. However, we will be able to order the groups against the mutations.

With these data both PRIM and ChL predict confluent evolutionary trajectories (Supplementary Figures 19). In Figure 5 we show PRIM's direct acyclic graph and Edm's tree. Both models predict *branched tumor evolution* and high ITH among the subclonal populations, consistently with the phylogenetic analysis carried out in [66].

First, the clonal lesions – the clonal root – trigger the first expansions of this tumor, with mutations in the key colorectal drivers APC, KRAS, PIK3CA and TP53 [31]. These biomarkers are ubiquitous, and could not be used to disentangle the mutational spectrum of the primary tumor from the metastatic lesions, in accordance with [67].

Second, the models identify distinct branches outgoing from the trunk, which discriminate the different subclonal evolutions. In both models one subclonal trajectory is initiated by a *stopgain SNV* in the DNA damage repair gene ATM. Edm, in particular, characterizes region P3-1 by a subsequent accumulation of INHBA and CDKN2A nonsynonimous mutations, whereas P3-3 by SMAD4 (stopgain SNV) and KMT2C (frameshift). Conversely, PRIM infers a more complex model, in which two confluent trajectories anticipate common late mutations in different regions: mutations of either INHBA *or* of TGFBR2 may precede mutations of CDKN2A in region P3-1, whereas in region P3-3 alterations of either INHBA *or* of SMAD4 might precede alterations of KMT2C. Interestingly, the alterations of CDNK2A might point to a *cell cycle arrest hallmark* for this tumor. Notice that the model inferred via PRIM exactly fits in scenario (*iv*), which could not be identified with canonical phylogenetic approaches.

Third, in both models the *subclonal metastatic* expansion is originated by a stopgain SNV in GNAQ, anticipating mutations in SMAD4, SETD2, AR (i.e., the subclonal group) and PPP2R1A. The models suggest canonical convergent evolution (i.e., parallel) towards SMAD4 (a stopgain SNV in the primary tumor, and

a nonsynonimous mutation in the metastasis). The transducer of transforming growth factor-β superfamily signaling SMAD4 regulates cell proliferation, differentiation and apoptosis [68], and its loss is usually correlated with colorectal metastases [69]. AR is a transcription factor that regulates cell migration and inhibites hepatocellular carcinoma metastases [70]; its splice variants are known to promote metastasis in several tumor types [71]. Similarly, GNAQ is supposed be relevant in metastases development in certain tumor types [72]. PPP2R1A is a negative regulator of signal transduction, gene expression and cell cycle [73], and its mutation influences tumor-endothelium interaction in melanoma metastases [74]. Notice that many other genes that are supposed to characterize MSI-high progression are wildtype in this tumor, e.g., FBXW7, BRAF, ARID1A, FAM123B, etc., [31], as a further evidence of the high level of ITH even within the same tumor subtype.

We finally compared the ordering estimated by TRaIT to the predictions obtained by SCITE (Supplementary Figure 20). Both approaches predict the same formation of the metastatic lesion, yet some significant differences are present. First, SCITE predicts that the mutation of ATM triggers tumor initiation, prior to the mutations included in TRaIT's clonal group, which are ordered in a 34 events-long linear chain. Yet, this specific order has score equivalent to several other models (Supplementary Figure 20) and, thus, might be unreliable. Besides, in SCITE's model TGFBR2 is associated to region P3-1 (in accordance with PRIM, but not with Edm), GNAQ's stopgain is upstream to both P3-3 and L branches, and some relations appear in inverted temporal ordering, e.g., between SMAD4 and KMT2C. Finally, by construction, SCITE can not infer any confluent evolutionary trajectory.

Conclusions

The increasing availability of high-resolution multi-sample sequencing data allows one to study ITH at an unprecedented resolution, to understand origination and development of tumors both at the genotype and phenotype level, and to better stratify and treat cancer patients.

Multi-region and SCS data harbours signals that can be informative of different aspects of tumor evolution. In fact, several techniques have been developed that either deconvolve clonal signatures, determine the ordering of growing clones or accumulating mutations, or estimate clonal fractions and cellular prevalence. The concerted application of these techniques allows to draw complex pictures of cancer evolution. As we show for a triple negative breast cancer, one can use the same dataset to detect *both* the signatures of the prevalent clones, *and* to infer the temporal precedence (i.e., ordering) of mutations that generated them. With the right tools, one can hence understand *which* clones are annotated in the data, and *how* they were shaped by evolutionary pressures.

The majority of techniques that perform such analyses ground their roots in standard phylogenetic theory, or in some of its cancer-specific derivations. These techniques are very effective, but sometimes they also implement a noteworthy deal of technical assumptions regarding sequence substitution models, alleles fixation, noise or search scheme etc. As a consequence, it could be hard to quantify how much the final predictions are shaped by the model and its assumptions, or actually suggested by the data. For instance, complex noise-learning

models to leverage the imbalances of SCS data might resolve the ordering of clonal mutations in arbitrary ways. This manifests as long trunks whose actual order can not be estimated from current data, and the inclusion of subclonal mutations in dubious positions in the trunk (Supplementary Figure 19).

Similarly, when one seeks for a maximum parsimony phylogenetic tree of tumor evolution, several equivalent-scoring solutions could be returned. When that happens, one has to implement disambiguation heuristics to select *one* output model [39, 40]. This could be one of the computed trees, or a new tree that is a combination of those (e.g., a bootstrap consensus [75]). Despite these routines are often adopted, they are somewhat arbitrary and some deal of care should be warned.

In this paper, we deviate from phylogenetic methods and present the TRaIT computational framework, whose methods give statistically robust estimates of mutational orderings. Our models are simple, and can be interpreted straightforwardly: if an edge connects two mutations (i) it resolves their temporal ordering and (ii) the mutations are statistically dependent. Both conditions are estimated from data without using complex inferential models, and assessed with p-values. Statistical assessment of models' confidence can be obtained by usual bootstrap or cross-validation approaches [31].

The simplicity of our framework has other advantages, both from an evolutionary and a computational point of view. First of all, TRaIT's models can account for any variable that can be annotated in a tumor sample. Thus, with TRaIT one can introduce high-level information on pathways, hallmarks, phenotypic-triggering lesions or epigenetic states (e.g., *methylations*), as long as they are *persistent* during tumor evolution. Inclusion of these information in traditional phylogenetic methods that work with sequences seem harder. Second, TRaIT implements four optimal (i.e., polynomial-time) algorithms that look for different types of signals in the sequencing data. These can be used to investigate whether data suggest the presence of confounding factors, or if the tumor originated from multiple cells, or if late mutations are selected by more than one evolutionary trajectory (i.e., a generalization of convergent evolution at the gene-level [39][2]). To the best of our knowledge, approaches based on phylogenetic theory can not detect these kind of signals, as they are limited to estimating single trees from data. Thus, with TRaIT's algorithms one can test a broad set of hypotheses on tumor evolution as we show in a colorectal cancer case study where we find convergent selection towards CDKN2A, which might point to a cell cycle arrest hallmark for this tumor type.

The computational burden of our techniques is also limited, compared to standard Bayesian approaches (which, however, include an estimation of uncertainty within the model). We do not compute a full posterior over our estimates, but rather a Maximum A Posteriori model constrained by Suppes' conditions. These conditions impose minimum levels of significance to the ordering predicted by our models, and are enforced as *empirical Bayes priors*. In light of the increasingly available data – especially from SCS – this leads to important scalability properties, whereas full Bayesian computations might become more demanding. Our methods accommodate low-effort parallel implementations [76], which we provide in the TRONCO tool for TRanslational

[2]We refer to Supplementary Figure 4 and Supplementary Tables 4,5 and 7 for a discussion of the statistical complications arising from such a generalization.

ONCOlogy [63, 64].

Our methods could be improved in several ways. For instance, we could pair bulk sequencing samples to either SCS or multi-region inputs; in fact, the combination of these has been recently shown to improve the estimation of the mutational ordering [77]. Furthermore, we could extend our framework to infer, besides mutational orderings, clonal signatures and architectures, in the attempt of defining a unified framework for cancer evolutionary inference.

From a broader perspective, our methods build on our earlier contributions on tumor evolution from single-sample bulk sequencing data [29–31]. These causal models allowed us to define the first automatic pipeline to quantify intertumor heterogeneity across multiple patients [31]. In this context, however, since we observe data from just one patient, we can not generalize our finding to causal claims (see Supplementary Material).

To conclude, we advocate the use of our methods as complementary approach to phylogenetic tools studying tumor evolution, in a joint effort to better quantify the extent of ITH.

Methods

Mathematical details of all TRaIT's algorithm are provided as Supplementary Material.

Suppes' probabilistic causation [65]. Let $p(\cdot)$ be multinomial estimates of the probabilities in D. For every pair of variables x and y in D, x is a plausible cause of y if

$$p(x) > p(y) \quad \textit{and} \quad p(y \mid x) > p(y \mid \neg x)\,. \tag{1}$$

The former condition acts as an *infinite sites assumption*, as we are assuming that *lesions are persistent* (no back-mutations). So, we estimate temporal precedence by marginal probabilities. The latter implies condition statistical dependence: $p(x, y) \neq p(x)p(y)$ [29]. For an edge to be part of our models, *both* conditions must be satisfied. When this is not the case, an edge is included and a non-significant p-value returned (see below). By iterating this approach, we can create models.

This tool is the core ingredient of successful causal approaches for cancer evolutionary inference [30]. It represents a necessary but not sufficient estimator of selective advantage, and combined with a statistical frameworks to disentangle true from spurious associations, can detect selection [31]. With data from a single patient, we limit its power to predict just temporal orderings (see Supplementary Material).

Working scenarios (Figure 1). Theres is a huge deal of variability in cancer data types, in cancer ITH, as well as in our ability to call mutations etc. Besides, several aspects of cancer evolution are yet undeciphered, so we considered four working scenarios representative of different biologically and technologically-motivated assumptions, and defined corresponding algorithms.

The simplest setting is (i) when all D's variables are *involved in the progression*. Then, we generalize this to when (ii) some of D's variables are annotated

in D, but irrelevant to tumor progression (e.g., calling uncertainty or other *confounding factors*). Besides, we account for a (iii) a tumor with multiple cells of origin, and we aim at identifying multiple independent models from a unique dataset. The fourth case is that of a tumor that shows (iv) selective pressures that converge towards variable x. It seems reasonable to consider (i, ii) more common than (iii, iv).

Algorithms (Figure 2). TRaIT's algorithms use a *non-parametric bootstrap strategy* to assess Suppes' conditions among variables pairs, and include them in a *direct graph* G. Then, four different strategies can compute, from G, a model. Output models can be interpreted as *Suppes-Bayes Causal Networks* [78–80], an extension of Bayesian Networks, with *maximum likelihood estimates* of the parameters θ [81] – bearing in mind that usually such models are used in truly causal approaches. The output is *the Maximum A Posteriori probabilistic model* that best explains D.

The algorithms are inspired by $(i - iv)$, which require us to infer (i) a mutational tree T, (ii) T and some detached nodes for the variables identified as confounding, (iii) a set of trees, $\{T_i\}$, usually called a *forest* and (iv) a *direct acyclic graph* G (since confluent trajectories lead to a node with multiple incoming edges).

TRaIT implements two algorithms to infer trees: Edm (Edmonds), Gbw (Gabow), based on *weighted directed minimum spanning tree* reconstruction. These algorithms scan G to identify the T that maximizes the edges' weights, which are computed via information-theoretic measures of the degree of association of variables – e.g., (pointwise) *mutual information*. Edm and Gbw differ from the way they order *strongly connected components* [82, 83] that appear in G because of finite sample bias. Computationally, Gbw is more expensive and general than Edm.

Two additional algorithms, ChL (Chow-Liu), PRIM, are available to infer direct acyclic graphs. ChL is a Bayesian model-selection method to factorize a joint distribution over the input variables [84]. PRIM is the equivalent to Edm for undirected structures, is applied by rendering G undirected, and weighting it with mutual information (which is symmetric). By assigning *a posteriori* an ordering to the undirected spanning trees that is consistent with the variables' frequency, we can retrieve confluent relations.

Detection of k independent progressions is a feature available for all algorithms, as it is enforced by the bootstrap when G has k disconnected components. Each group describes evolutions as triggered by multiple initiation cells. Thus, by G's estimation and by picking the proper TRaIT's algorithm, one can easily cover scenarios $(i, \ldots, iv)$.

Complexity. We observe that *all* TRaIT*'s algorithms are optimal polynomial-time algorithmic solutions to each of their corresponding combinatorial problems.* Thus, they *scale well with sample size*, a problem sometimes observed with Bayesian approaches that cannot compute a full posterior and need to sample. TRaIT's algorithms, however, do not have a rich description of uncertainty since they return a single model, but can be however paired with a posteriori forecasts' assessment strategies (e.g., cross-validation/ bootstrap) rather easily [31].

Data types. TRaIT's algorithms work with both SCS and multi-region data. We expect D to contain *noisy observations* of the *unknown true genotypes* (Figure 1). The algorithms can be informed of the usual *false positives and negatives* rates $\epsilon_+ \geq 0$ and $\epsilon_- \geq 0$, respectively. This adds no overhead to the computation, but prevents to learn noise rates from D, as it is instead possible with techniques as SCITE [57]. Since the algorithms show stable performance for slight variations in the input noise rates, avoidance of complex noise-estimation schemas seems a plus, especially when reasonable estimates of ϵ_+ and ϵ_- are known a priori. This strategy is also used in OncoNEM [59].

For SCS with missing data we use a standard *Expectation Maximization* approach to input missing values; we repeat it n times, and then perform inference and select the MAP best model out of the n trials.

Author's contributions

DR, AG and GC designed the algorithmic framework. DR, LDS and GC implemented the tool. LDS carried out the simulations on synthetic data. Data gathering was performed by DR, AG, LDS and GC. All the authors analyzed the results and interpreted the models. DR, AG, BM, MA and GC wrote the original draft of the paper, which all authors reviewed and revised in the final form.

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Figures

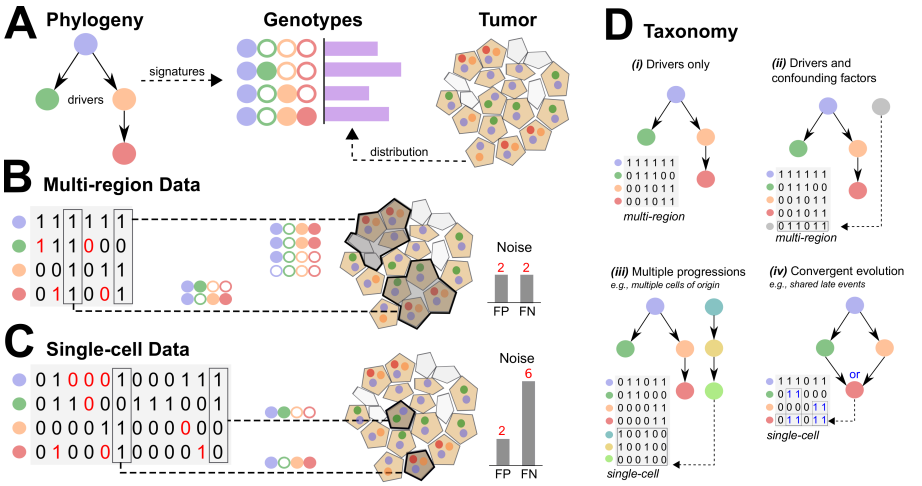

Figure 1: **A.** A phylogenetic model of tumor progression describes the order of accumulation of somatic mutations, CNAs, etc. The model describes a set of possible genotype signatures, which are observed with an unknown spatial and density distribution in a real tumor. **B.** Multi-region bulk sequencing processes a signal mixed from different tumor subpopulations, with potential contamination of non-tumor cells. Thus, a sample will be likely annotated with lesions from different tumor lineages (green, red), creating spurious correlations in the data. In this case, we expect the rate of false positives and negatives in the calling to be symmetric. **C.** If we sequence genomes of single cells we can, in principle, have a precise signal from each subpopulation. However, the inference with these data is made harder by high levels of asymmetric noise, and errors in the calling. **D.** We are interested in studying temporal models of cancer progression in 4 possible scenarios. (i) when all annotated mutations are related to the progression, (ii) when data harbours confounding factors, (iii) when a tumor might have multiple cells of origin and, accordingly, multiple independent progressions and (iv) when independent evolutionary trajectories converge toward a certain mutation (i.e., a confluence). Case (iv) is more general than parallel evolution (i.e., distinct mutations on the same gene).

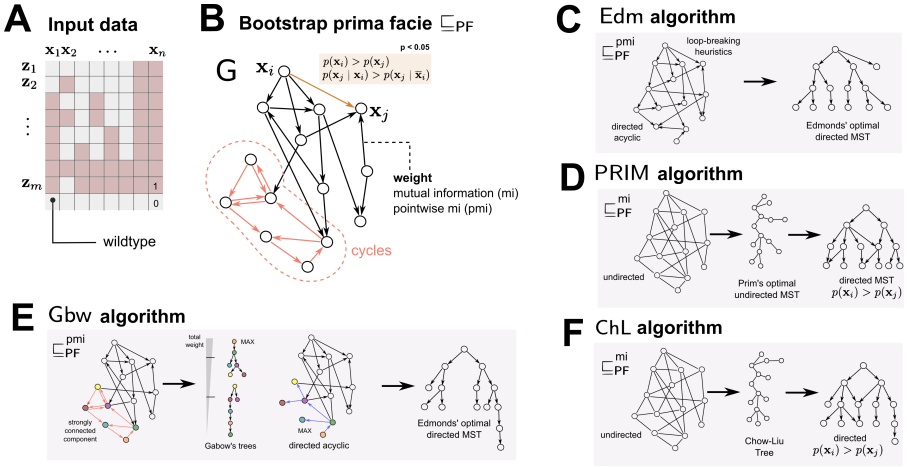

Figure 2: **A**. TRaIT's input data is a binary matrix that stores the presence/absence of a variable in a sample (e.g., a mutation, a CNA, or a persistent epigenomic lesion). Some of these observations harbour false positives and negatives (noise). **B**. We estimate via bootstrap the *prima facie* ordering relation $\sqsubseteq_{\mathsf{PF}}$ that satisfies Suppes' conditions for probabilistic causation, here used as estimators of temporal orderings. This, in turn, induces a graph G over variables $\mathbf{x}_i$. G can be weighted by information-theoretic measures for variables' association. **C.** we weight G with pointwise mutual information (pmi), and we use an heuristic that makes G acyclic by removing less confident edges (see [30]); then we use Edmonds' optimal solution for minimum directed spanning trees. **D.** if we weight G with mutual information (mi) and disregard edges' orientation, we can use Prim's optimal solution for minimum undirected spanning trees. To find a direction for each edge, we use mutations' frequencies. **E.** if we use Gabow's optimal solution for path traversals of cyclic component, we can detect the best tree that makes G acyclic. Then, we can again use Edmonds' algorithm for spanning trees. **F.** A Bayesian optimal mode-selection strategy can compute the Chow-Liu tree that induces the distribution with minimum divergence from the true one. In this case, we process G as in PRIM to detect edges' direction.

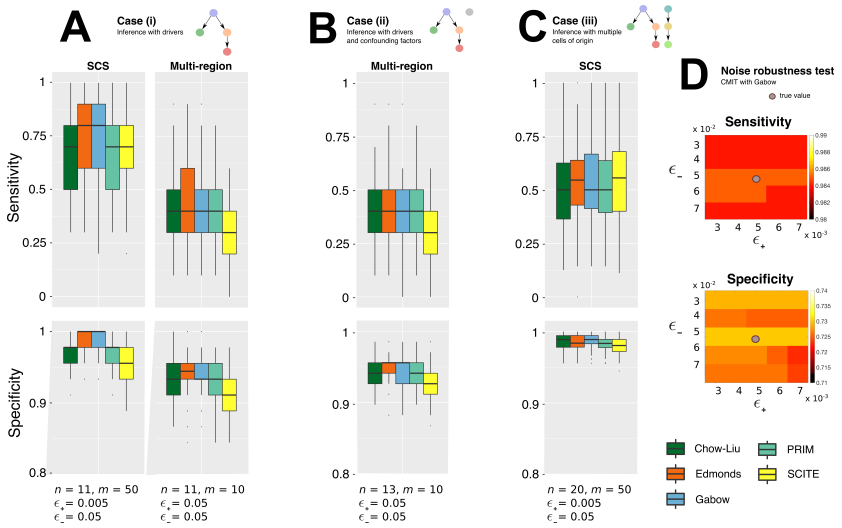

Figure 3: We estimate from simulations the rate of detection of true positives (*sensitivity*) and negatives (*specificity*), visualized as *box-plots* from 100 independent points. We compare TRaIT's algorithms and SCITE, the state-of-the-art for mutational trees inference. For each data type, here we show here a mild-noise setting with canonical sample size: in SCS data noise is $\epsilon_+ = 5 \times 10^{-3}; \epsilon_- = 5 \times 10^{-2}$, in multi-region $\epsilon_- = 5 \times 10^{-2}$. Extensive results for different models, data type, noise and sample size are in Supplementary Figures 3–14. **A.** Here we use a generative model from [58] (Supplementary Figure 5). (left) SCS datasets with $m = 50$ single cells, for a tumor with $n = 11$ mutations. (right) Multi-region datasets with $m = 10$ spatially separated regions, for a tumor with $n = 11$ mutations. **B.** We augment the setting in A-right with 2 random variables (with random marginal probabilty) to model confounding factors, and generated SCS data. **C.** We generated multi-region data from a tumor with $n = 21$ mutations, and a random number of 2 or 3 distinct cells of origin to model independent progressions. **D.** Spectrum of average sensitivity and specificity for Gbw algorithm estimated from 100 independent SCS datasets sampled from the generative model in Supplementary Figure 5 ($m = 75$, $n = 11$). The true noise rates are $\epsilon_+ = 5 \times 10^{-3}; \epsilon_- = 5 \times 10^{-2}$; we scan input ϵ_+ and ϵ_- in the ranges: $\epsilon_+ = (3, 4, 5, 6, 7) \times 10^{-3}$ and $\epsilon_- = (3, 4, 5, 6, 7) \times 10^{-2}$.

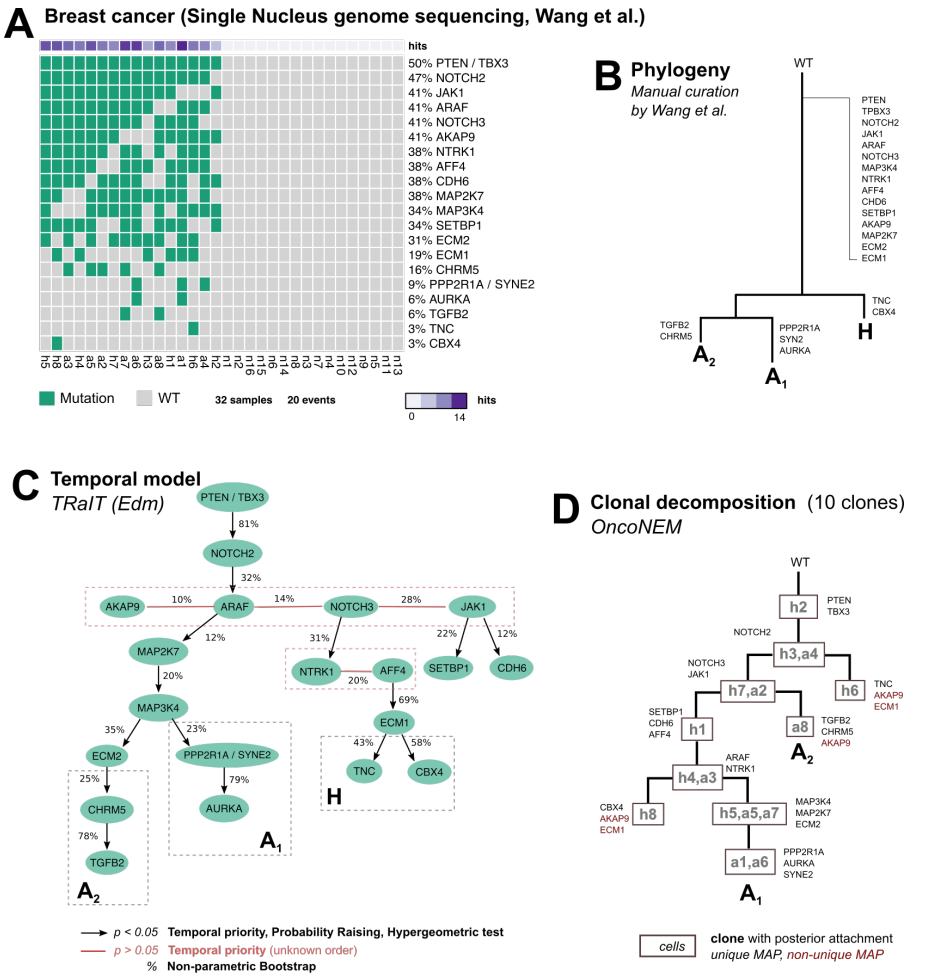

Figure 4: **A.** Input data from single-nucleus sequencing of a triple-negative breast cancer [50] (32 cells). The rate of missing values for this dataset is very low (around 1%), allelic dropout has rate 9.73×10^{-2}, and false discovery 1.24×10^{-6}. **B** Manually curated phylogenetic tree estimated in [50]. Mutations are annotated to the trunk if they are ubiquitous across cells, and detected also in a bulk control sample [50]. Subclonal mutations are those appearing only in more than one cell. **C**. Temporal mutational tree obtained with Edm algorithm; p-values are obtained by 3 tests for Suppes' conditions and overlap (hypergeometric test), and edges annotated with a posteriori non-parametric bootstrap scores (100 estimates). For these data, all TRaIT's algorithms return trees (Supplementary Figure 16), consistently with the manually curated phylogeny (A). Most edges are highly confident ($p < 0.05$), expect for groups of variables with the same frequency which have unknown ordering (red edges). The ordering of mutations in subclones $\mathtt{A}_1$, $\mathtt{A}_2$ and tumor initiation has high bootstrap estimates ($> 75\%$). **D**. We perform a concerted analysis to estimate both clones' signatures and their formation, at least for mutations with a clear statistical signal in these data. We do this by computing a clonal tree with OncoNEM, which predicts 10 clones. Mutations are assigned to clones via *maximum a posteriori* estimates. The mutational ordering of the early clonal expansion of the tumor, which involves mutations in PTEN, TBX3, NOTCH2, is consistent among both models. The same happens for most of the late subclonal events, e.g., mutations in MAP2K7, MAP3K4, PPP2R1A, SYNE2 and AURKA in subclone A1. However, the temporal ordering of intermediate events has weaker support as they have the same marginal probability, and any permutation of their ordering would be equivalent for our method.

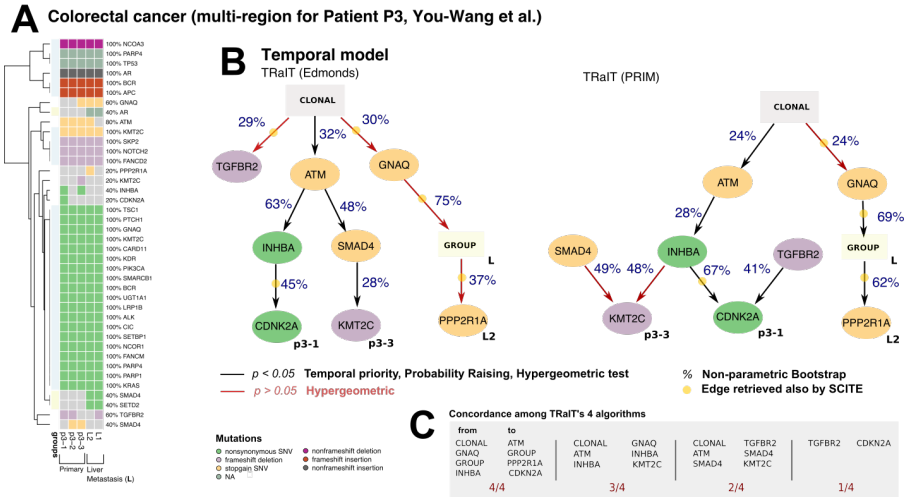

Figure 5: **A**. Multi-region sequencing data for a MSI-high colorectal cancer [66], with three regions of the primary cancer: `p3-1`, `p3-2` and `p3-3`, and two of one metastasis: `L-1` and `L-2`. To use this data with TRaIT we merge mutations that have the same signature across all regions, obtaining a *clonal* group (light blue) including 34 mutations and *sublclonal* group (light yellow) including: non-synonymous SNVs of SMAD4 and SETD2, and non-framshift insertion of AR. **B**. Models obtained by Edm and PRIM algorithms, with their confidence annotated and the overlap in the predicted ordering obtained by SCITE. PRIM predicts convergent evolution towards a non-synonymous mutation in CDKN2A, which is also predicted by ChL (Supplementary Figure 18). All edges, in all models, are statistically significant for Suppes' conditions (temporal precedence and selection strengths). **C.** Four of the predicted ordering relations are consistently found across all TRaIT's algorithm, which gives a high-confidence explanation for the formation of the L2 metastasis. This finding is also in agreement with predictions by SCITE (Supplementary Figure 19).