

RESEARCH

LACE 2.0: an interactive R tool for the inference and visualization of longitudinal cancer evolution

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Abstract

Background: Longitudinal single-cell sequencing experiments of patient-derived models are increasingly employed to investigate cancer evolution. In this context, robust computational methods are needed to properly exploit the mutational profiles of single cells generated via variant calling, in order to reconstruct the evolutionary history of a tumor and characterize the impact of therapeutic strategies, such as the administration of drugs. To this end, we have recently developed the LACE framework for the Longitudinal Analysis of Cancer Evolution.

Results: The LACE 2.0 release aimed at inferring longitudinal clonal trees enhances the original framework with new key functionalities: an improved data management for preprocessing of standard variant calling data, a reworked inference engine, and direct connection to public databases.

Conclusions: All of this is accessible through a new and interactive Shiny R graphical interface offering the possibility to apply filters helpful in discriminating relevant or potential driver mutations, set up inferential parameters, and visualize the results. The software is available at: github.com/BIMIB-DISCo/LACE.

Keywords: single cell; longitudinal; clonal analysis; shiny; interface

Background

We present LACE 2.0, a new R package to analyze single-cell (SC) mutational profiles generated from *longitudinal* sequencing experiments of cancer samples, which are increasingly becoming available due to the continuous advances in biotechnology. Robust computational frameworks for SC sequencing data are needed to mitigate the impact of technology-related errors, as well as that of sampling limitations, so as to deliver a fine characterization of intra-tumor heterogeneity and to possibly cast a light on the mechanisms underlying drug resistance and relapse under a variety of conditions and experimental setups. In recent years, excellent tools for the inference of clonal/mutational trees for SC experiments, but incapable of handling longitudinal data [1, 2], or longitudinal bulk data[3] have been proposed to investigate the mutational evolution of cancer. LACE 2.0 embraces the possibility to analyze the development of the disease using SC data from longitudinal studies and is capable of deriving a clonal tree tagged with time coordinates coherent with the sampling temporal order.

In this context, the original version of LACE was designed to exploit binarized mutational profiles, generated via the application of standard pipelines for variant calling to either DNA- or (full-length) RNA- single-cell sequencing experiments,

performed at multiple time points. To do so, LACE maximizes a weighted likelihood function by solving a Boolean matrix factorization problem via MCMC sampling [4], and proved to be accurate and robust even with high rates of noise and data-specific errors. The latest LACE version (2.0) extends and enhances the original version in several ways. Thanks to a completely new **Shiny** app interface [5], LACE 2.0 includes: (a) an easy-to-use module for data preprocessing and organization, which allows one to test and adjust quality and relevance filters, and to annotate gene variants; (b) a module for the interactive visualization of the inferred cancer evolution models, which returns both the *longitudinal clonal tree* and the *fishplot*, and allows one to inspect the model (i.e., clones and temporal relations), also by querying external databases such as **Ensembl** [6]. Importantly, LACE 2.0 requires a limited set of parameters, which are sufficient to mitigate the source of noise and infer reliable models of cancer evolution (see [4] and [7] for further details).

1 Implementation

LACE 2.0 exploits binarized mutational profiles (1 if a mutation is present in a given cell, 0 if it is absent, NA for low coverage) generated by calling variants from SC DNA- or full-length RNA-seq experiments of cancer samples collected at multiple time points, usually before and after therapy. LACE 2.0 returns as output a *longitudinal clonal tree* [4], in which nodes represent *clones*, i.e., groups of single cells with the same genotype, and edges represent either *parental relations*, e.g., clone B originates from clone A thanks to the acquisition of a new mutation, or *persistence relations*, e.g., clone C is present at both time points 1 and 2 .

LACE 2.0 is built as a shiny app allowing the user to apply filters, perform operations on the data and visualize the results. The interface is organized in 8 modules represented by tabs:

- Project Creation
- Metadata Info
- Gene Variant Annotations
- Quality Filters
- Single Cell Sampling Depth
- Variant Filters
- Inference
- Longitudinal Display

Stepping through each of these modules one can select mutations according to distinct criteria in order to generate the input for the inferential routines, set parameters for the derivation of the longitudinal relations among clones and display the most likely clonal tree and hyper parameters describing the time evolution of the samples.

1.1 Inputs

LACE 2.0 requires as input the result of standard alignment and variant calling pipelines, applied to either SC DNA-seq or (full-length) RNA-seq data. Concerning the former, even though whole-genome and whole-exome SC sequencing experiments may be appropriate for genotyping, they are rarely available due to the high costs, so targeted genome sequencing appears to be a sounder option to this end. Also

full-length SC RNA-sequencing experiments (e.g., SmartSeq) can be employed in LACE 2.0, despite the known issues related to coverage, which is typically low and limited to transcribed regions [7]. Yet, this data type is extremely diffused and has the notable advantage of providing a natural mapping between the genotype and the gene expression of single cells. The first preprocessing step (*not* included in LACE 2.0) consists in aligning the library to a reference genome and perform variant calling. This can be done using any of the widely used pipelines (see [8]). Using LACE 2.0, single variants can be then annotated using Annovar [9] as a back-end. LACE 2.0 takes as input the BAM and the VCF files so generated, and allows the user to set different quality and relevance filters with a simple interactive form of the Shiny interface.

1.2 Project Creation

In order to organize analyses with different parameters and to store intermediary steps, each inferential experiment with all the setting are saved in a subfolder named after the project id which is selected by the user. At the moment a project is created, default configuration settings are generated marking the subfolder as part of the LACE 2.0 analysis. If the selected subfolder is already a LACE 2.0 project, the app recognizes it and loads with the last configuration used. A project is saved after each user interaction with the interface, and it can be reloaded directly by selecting the project folder or by the sidebar where the latest project names are displayed.

1.3 Metadata Info

In order to perform clonal analyses, it is required to provide some information about the experiment such as the cell IDs which are used to retrieve the VCF/BAM files and the sample name each cell belongs to. This metadata is generally included with the experimental sequencing data as part of the library preparation. LACE 2.0 accepts tsv/csv tabular formats with headers and rds standard tabular data R format. The metadata file can include more information relative to the experimental setup and the platform used.

After selecting the single cell ID and the sample name columns, the user can reorder the samples in chronological order. This is the only step where time information is provided during the analysis, and it will be used in post inferential computation to generate the longitudinal clonal tree and the ordered sets of clonal prevalences.

1.4 Gene Variant Annotations

Under the ISA assumption, clonal analysis is performed by grouping cells in clades with sets of similar mutations and by linking them via inclusion relation of mutation sets. In order to avoid excessive partition of cells over variants with unspecified roles in the phenotypical cascade, as in diseases with high mutational rate, it is useful to annotate mutations so as to select only relevant classes. LACE 2.0 performs annotations of variant calling data using Annovar as back-end. Each mutation of each cell is annotated based on the annotation database used. Annovar provides a database for the human species useful for tagging cancer mutations and, whenever possible, providing their functional effect.

If Annovar's Perl scripts are available in the OS standard binary paths, LACE 2.0 automatically detects them and sets their path; otherwise, the user should provide the folder containing the Perl scripts. The user should also provide the database to use for the annotation and the folder containing the variant calling files in VCF format obtained by standard pipelines.

1.5 Quality Filters

All types of SC sequencing data is characterized by various sources of noise which depends on the technology used. Detected mutation might be characterized by low quality score or low statistical power. Some mutations can be neglected, while others might cause relevant effects, especially on exonic regions where variations can result in changes of the translational process and modify their functional form.

In order to avoid small and possibly spurious fluctuations on sequencing data, variants can be filtered if they have low supporting evidences. The user can set the minimum values for:

- The number of reads supporting the alternative alleles in a cell. Due to sampling noise, cells not showing enough alternate reads for a mutation are considered not mutated at that site and are not counted.
- The frequency of the minor allele for each referenced SNP included in a default global population. To assess the significance of somatic mutations, if variants are absent in control subjects or the variants have very low MAF (e.g., < 0.005), they are marked as somatic mutations. In comparison, if the MAF in the general population is big enough (e.g. > 0.01), they are considered possible deemed polymorphic or benign variants [10, 11, 12].
- The cell frequency per sample showing the mutation at the same site. Mutations which are not supported by a minimum amount of cells at each sample can be excluded, if necessary, due to heterogeneity and fluctuations.

Furthermore, the user can choose which functional exonic variation should be considered or neglected. For example, there are cases in which unknown and synonymous mutations in exonic regions are disregarded because their effect cannot be explicitly related to the experimental condition. All possible variant functions and their explanations are resumed in Table 1.

At the end of this module, a time-indexed binarized matrix representing the remaining mutations for each the cell in each sample is generated. For any given cell, SNVs exceeding the number of reads supporting the alternative alleles threshold will be set to 1, or 0 otherwise.

1.6 Single Cell Sampling Depth

The number of reads per variant site represents an optimal filter to retrieve relevant mutations. Read depths along the sequences are usually not provided in standard alignment or variant calling pipelines. The user should provide the Samtools Suite [13] executable folder location in case LACE 2.0 cannot find back-end in the OS standard folder. Furthermore, the folder with the longitudinal single cells aligned data should be provided as input for the computation. To be noted, aligned files must have a case insensitive ".bam" extension, and only filenames coinciding with the cell IDs provided in the metadata info are analyzed, while other files are neglected.

Table 1 Variant calling types of annotation supported by the back-end [9].

| Annotation | Explanation |
|----------------------------------|---|
| frameshift insertion | an insertion of one or more nucleotides that cause frameshift changes in protein coding sequence |
| frameshift deletion | a deletion of one or more nucleotides that cause frameshift changes in protein coding sequence |
| frameshift block substitution | a block substitution of one or more nucleotides that cause frameshift changes in protein coding sequence |
| stopgain | a nonsynonymous SNV, frameshift insertion/deletion, nonframeshift insertion/deletion or block substitution that lead to the immediate creation of stop codon at the variant site. For frameshift mutations, the creation of stop codon downstream of the variant will not be counted as "stopgain"! |
| stoploss | a nonsynonymous SNV, frameshift insertion/deletion, nonframeshift insertion/deletion or block substitution that lead to the immediate elimination of stop codon at the variant site |
| nonframeshift insertion | an insertion of 3 or multiples of 3 nucleotides that do not cause frameshift changes in protein coding sequence |
| nonframeshift deletion | a deletion of 3 or multiples of 3 nucleotides that do not cause frameshift changes in protein coding sequence |
| nonframeshift block substitution | a block substitution of one or more nucleotides that do not cause frameshift changes in protein coding sequence |
| nonsynonymous SNV | a single nucleotide change that cause an amino acid change |
| synonymous SNV | a single nucleotide change that does not cause an amino acid change |
| unknown | unknown function (due to various errors in the gene structure definition in the database file) |

Due to the fact that retrieving depth for all SC at variant sites is computationally expensive to retrieve, the procedure is only performed on sites passing the quality filters previously set.

1.7 Task Manager

LACE 2.0 tries to limit time consuming steps using a task manager based on file timestamps specifically developed for the purpose of reducing unnecessary heavy computations. More precisely, a computation step is defined by the input files, the output files and the required parameters stored in a temporary configuration file. If a task is a bijective function, the task manager checks if input file timestamp < configuration file timestamp < output file timestamp, and only for those files for which the relations do not hold true, the task is recomputed. If a task is an injective or a surjective function, the task manager controls if all input file timestamps < configuration file timestamp < all output file timestamps, and the task is recomputed only if the relations do not hold true.

This is very useful when tasks are chained, and the user modified parameters of downstream steps or if the user decided to add new acquired samples in the analysis (e.g., annotation and depth at variant sites can require a long computational time).

1.8 Variant Filters

Not all mutations are distinctive of the disease or experiment understudied. Identifying relevant and driver variants allows to reproduce a more significant longitudinal clonal tree. The user can select a set of filters based on gold standards and other analyses such as:

- The minimum number of reads at given mutation site. Depth represents the strength of the signal measured, and this filter marks previously identified

mutations based on read depth at the single cell level. Where the signal falls below a given threshold, the corresponding mutations in a cell are less reliable and set to 'NA'.

- The maximum number of missing data per gene. If a mutation is marked as 'NA' in too many cells, the same knowledge on the supposed mutation site becomes more fuzzy; hence, it is prone to be marginal.
- The minimum median depth per locus. This filter ensures the site is sufficiently measured given the set of experiments sampled.
- The minimum median depth supporting the mutation. Similarly, the signal supporting the mutation should be sufficiently measured in the whole set of cells included in the analysis.
- Subset of known genes. If there are genes associated with treatments or are drivers for the disease or relevant for the understudied longitudinal experiment, they can be included directly by the user. The proposed list of gene names are obtained from the list of mutated genes which have passed the applied filters.

The final set of variant sites passing all filters are used to subset the binarized matrix obtained in the former module. Furthermore, values in the matrix corresponding to mutations falling in a position with a coverage below the above threshold are set to 'NA'. This produces a time-indexed binarized mutational matrix, which is processed by the inference engine. Finding the parameters to select variants is not an easy task, and the user might not know in advance how to choose the best set of filters. Hence, the user can apply the aforementioned filters and computations on VCF and BAM files to derive all the necessary aggregated information on the sampled cells. Afterward, the user is presented with an interactive live preview of variants passing the filters (including relevant parameters which can help in the selection) while values are changed.

1.9 Inference

The inferential tab allows the user to set all the parameters to solve the boolean matrix factorization problem and estimate the model parameters of the model using a MCMC to maximize the likelihood. The inferential step uses the following set of parameters:

- Learning rate
- False positive rates for each sample
- False negative rates for each sample
- Number of iterations in each MCMC search
- Number of restart for the MCMC 5 Early stopping number of iterations with no growing likelihood
- Number of parallel processes
- Random seed to recreate simulations
- Initialize the clonal tree randomly
- Marginalize the cell attachment matrix
- Keep equivalent solutions and return all of them
- Check indistinguishable event and remove them
- Estimate error rates of MCMC moves

The user can insert more than one false positive rate and one false negative rate value for each sample. During the inferential step, the maximization of the likelihood for each set of rates is performed, and the best results are returned.

1.10 Longitudinal Clonal Tree

The model adopted in LACE 2.0 (see also [4]) describes the clonal phylogenetic factorization of the underlying evolutionary process based on experiments measured at sequential time points under the ISA assumption and subjected to different sources of noise derived from sampling single cells sequencing data. The model can be explained by the following matrices:

- the single cell data matrix D where rows represent single cells, columns are all the mutations considered and entries are 1 when mutations are present and zero when absent,
- the cell attachment matrix C , where rows identify the single cells, columns are the clones and values are 1 for cells belonging to a clone,
- the phylogenetic matrix B , where rows are the clones, columns are the mutations, and it is a boolean triangular matrix equivalent to a tree with entries associating clones to mutations.

These matrices can be related via tensorial product by the following relation:

$$D = C \cdot B$$

which describes the model as a boolean factorization problem. The real sequenced data matrix G has the same shape of D and represents similar relations between sampled cells and measured variants such that values are 1 when a mutation is observed, zero when not or NA if there are no sufficient reads supporting the observations. Discrepancies between the optimal solution of the model describing the underlying experiment \tilde{D} and real data G ,

$$G \approx \tilde{D},$$

are characterized by depth and sequencing tech limitations used to measure each sample s at time t_s which generate false event occurrences with platform dependent false positive α_s and false negative β_s rates. Therefore, the Bayesian approach is determined by:

$$P(B, C|G) \propto P(G|C, B)P(B, C),$$

and, in the maximum likelihood is given by:

$$P(G|C, B) = P(G|\tilde{D}).$$

The model assumes that the longitudinal experiments are sampled from a unique generative phylogenetic matrix B ; consequently, without further time and cellular cooperation constraints, the likelihood with sample (platform) specific weights can be completely factorized by mutation and by single cell, rendering the model time

agnostic and tractable:

$$P(G|B, C) = \prod_s \left[\prod_{i=1}^{n_s} \prod_{j=1}^m P(D_{i,j}|G_{i,j}) \right]^{w_s},$$

where s is the sample, w_s is the sample specific weight, n_s is the number of cells in sample s and m is the index of the total set of mutations considered.

The clonal tree B and attachment matrix C maximizing the weighted likelihood function is computed on all time points, via a MCMC search scheme.

For each clonal matrix B exists one and only one clonal tree. The construction of the clonal tree from the clonal matrix B is given by the following set of operations:

- 1 a node of the clonal tree is generated for each row of B ,
- 2 a clone i is characterized by the acquired mutations which are the ones in the i -th row of B , B_i ,
- 3 the root of the clonal tree is the row k for which $\|B_k\|_1 = 1$ holds true,
- 4 given a clone i , its parent j among all the clones k with $\|B_k\|_1 < \|B_i\|_1$ is given by $j = \arg \min_k (\|B_i - B_k\|_1)$.

The last point is a direct consequence of the ISA assumption which excludes the possibility of back mutations.

A longitudinal clonal tree is an augmented clonal tree where nodes are duplicated for each sampling time t_s since the first time a clone has been observed, are labeled with increasing time tags going from t_s to the final experimental sampling time and same clones with consecutive times are joined.

Reproducing a longitudinal clonal tree from B is not a straight forward procedure. First, it should be noticed that observing a clone implies the usage of the knowledge coming from the clonal prevalence C . Second, if clones i is parent of clone j , than there is no guarantee the first observation time t_v of clone i and the first observation time t_w of clone j satisfy the relation $t_v \leq t_w$.

To avoid spurious appearance of child clones before parent clones, LACE 2.0 checks the time tags of the longitudinal clonal tree with the order of the sampling times. In case of time reversal among two time tagged nodes, it adds a node to the longitudinal tree representing the parent clone with prevalence zero and with the smallest time tag of the two considered nodes. Compared to LACE, LACE 2.0 returns the augmented longitudinal clonal tree with time tagged nodes corresponding to the measured sequential time points of the experiment without time reversal among parent-child nodes.

1.11 Longitudinal Display

After the inference, the Shiny interface of LACE 2.0 allows one to visualize and interact with the output cancer evolution model (Figure 2). In detail, LACE 2.0 returns: (a) the longitudinal clonal tree (generated via `Cytoscape.js` [14]), (b) the fishplot (generated via `TimeScape` [15]), (c) a summary table in which the optimal values of false positive α and false negative β rates, the number of selected mutations and the number of single cells are displayed, for each time point. All plots are interactive and the properties of the distinct clones at any time are shown via mouse-over (e.g., clonal prevalence, distinctive mutations, etc.). Importantly,

the genes hit by the mutations present in any given clone are highlighted and, by selecting their names, LACE 2.0 performs a direct query to the Ensembl gene annotation database [6], so as to facilitate any downstream analyses.

Figure 1 LACE 2.0 Project. LACE 2.0 allows to create new or load former and recent opened projects.

2 Discussion

Former releases of LACE do not use standard VCF and BAM formats as input of the clonal analyses. LACE [4] input was a list of samples for each time point such that an element is a matrix of dimension given by the number of cells pooled in the sample times with all the mutations observed and entries equal to 1, 0 or NA for observed, non-observed mutations or insufficient reads cases, respectively. On the other hand, LACE 2.0 allows the user to infer the SC longitudinal clonal tree starting from largely available formats which are outputs of many SC gold standard alignment and variant calling pipelines. Formerly, the derivation of putative drivers and relevant mutations was left out from the important inferential step, and users needed to adopt external independent procedures. Similarly, assignment of mutations to cells as noisy values were derived in pre-quality check routines. In the present release, starting from variant annotations and depth computation, the user can adopt various filters and selection thresholds in order to set SC mutations with low number of reads as not available data and discriminate significant mutations for the understudied longitudinal mutational process.

The output of LACE was the cell attachment matrix and the clonal tree for the longitudinal experiment represented by zeros whether clonal prevalences are null or 1 otherwise. This could have risen some problem in the reconstruction of a mutational tree with the relation imposed by the time constraints of the experiment. LACE 2.0 returns an augmented longitudinal clonal tree comprehensive of the time tagging and edges which takes into account the time order of the samples, hence correcting

Project: Small_dataset

Single cell metadata info

Information regarding the longitudinal single cell experiment. At a minimum, information about the cells' identifiers and the sample they belong to is required. The identifiers are used to identify the file names to include in the analysis and the samplings represent the time points. The cells used in the downstream analysis are the intersection between the identifiers present in the metadata and the BAM files stored in the drive.

You may have to create such a file from scratch. See the (i) button below.

If you have loaded one of the 'demo projects', changes on this tab are not considered.

Metadata file
..../Small_dataset/data_info.rds

Cell id column
Run

| Run |
|--------------|
| 1 SRR7424152 |
| 2 SRR7424153 |
| 3 SRR7424154 |
| 4 SRR7424155 |
| 5 SRR7424156 |
| 6 SRR7424157 |

Time column
Age

Sampling points
Drag the time points in chronological order (before the smaller times)

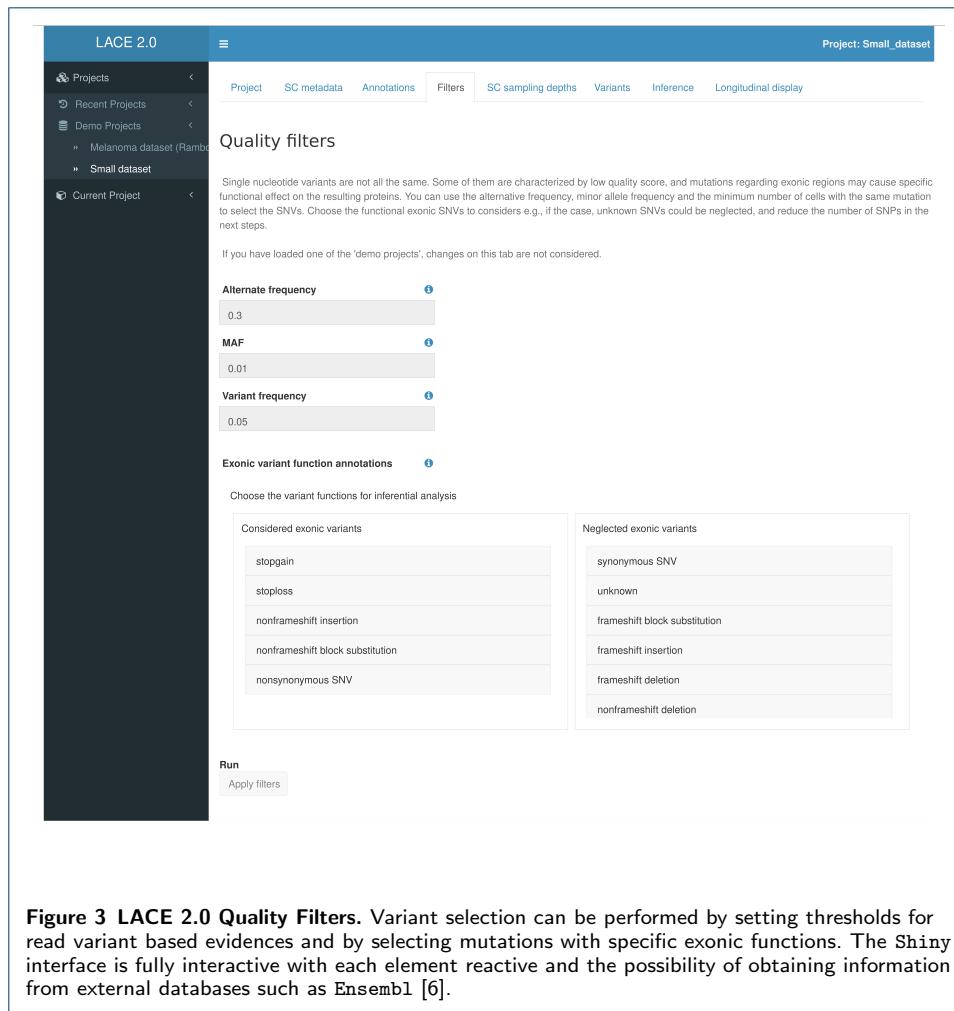
| Sampling point |
|------------------|
| before treatment |
| 4d on treatment |
| 28d on treatment |
| 57d on treatment |

Figure 2 LACE 2.0 Metadata Info. Select the SC id and the experiments' time coordinate and order.

for possible misrepresentations of clones' first appearance when clonal prevalences are zero.

3 Conclusions

In this work, we have presented the LACE 2.0 R package for the inference and interactive visualization of cancer evolution models from longitudinal single-cell sequencing experiments. This tool provides an important step in the direction of a wider diffusion of scalable and easy-to-use methods for single-cell analyses in cancer research, especially thanks to its graphical interface and the extremely limited programming skills required to perform any analysis. In fact, in a few well-defined data processing steps, it is possible to generate a high-resolution picture of the evolutionary history of a tumor and, most of all, explicitly assess the impact of any given therapy. Overall, we advocate the use of LACE 2.0 with already existing and highly-diffused SC RNA-seq datasets of patient-derived models, so as to naturally integrate current analyses on gene expression profiles with those on the underlying clonal evolution.



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Abbreviations

SC: Single cells
 SNVs: Single nucleotide variants
 ISA: Infinite allele assumptions
 BAM: Binary alignment map
 VCF: Variant call format

Availability of data and materials

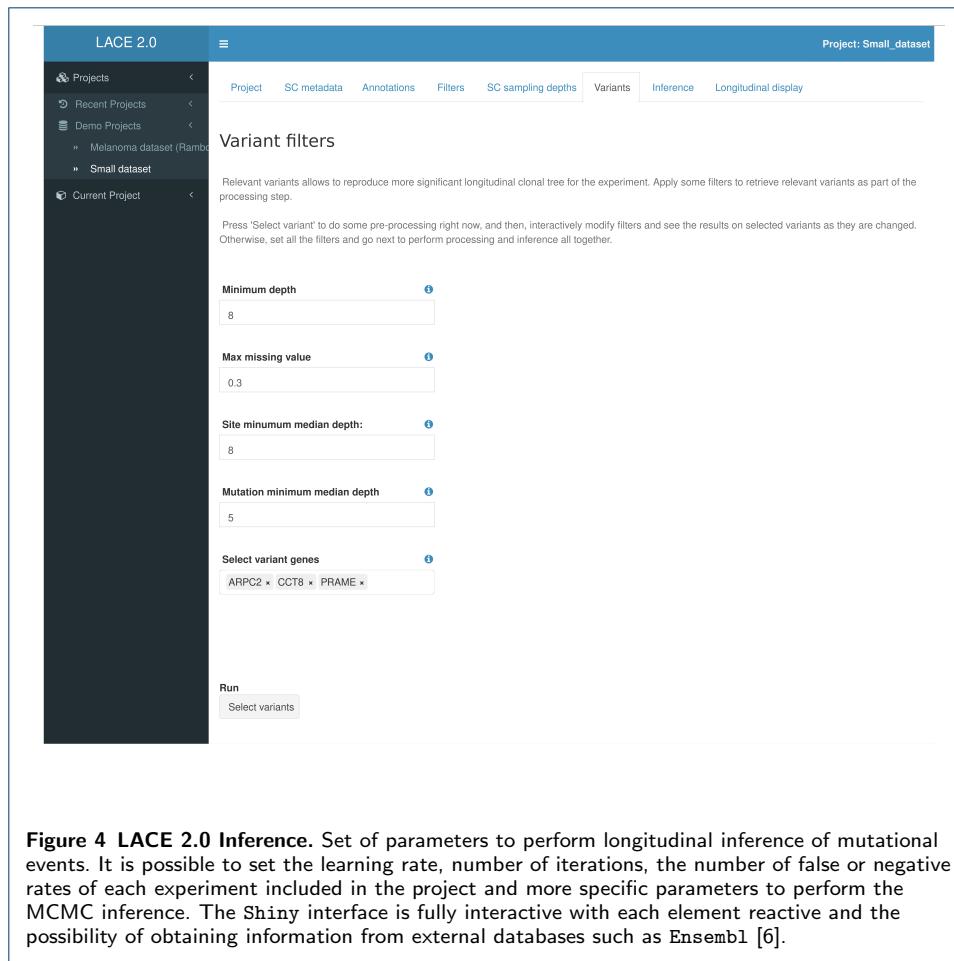
LACE 2.0 is available at: github.com/BIMIB-DISCo/LACE.
 The RNA SMARTseq2 accession number for the Melanoma dataset analysed during the current study is GEO: GSE116237.

Ethics approval and consent to participate

Not applicable

Competing interests

The authors declare that they have no competing interests.



Consent for publication

Not applicable

Authors' contributions

GA, NLSB, FA, AGr, MA designed the approach. GA, DM developed the methods and GA, NLSB, DM implemented them. GA developed the interface. GA executed data analysis. AGr, MA, GA supervised the work. All authors interpreted the results, discussed, drafted and approved the manuscript.

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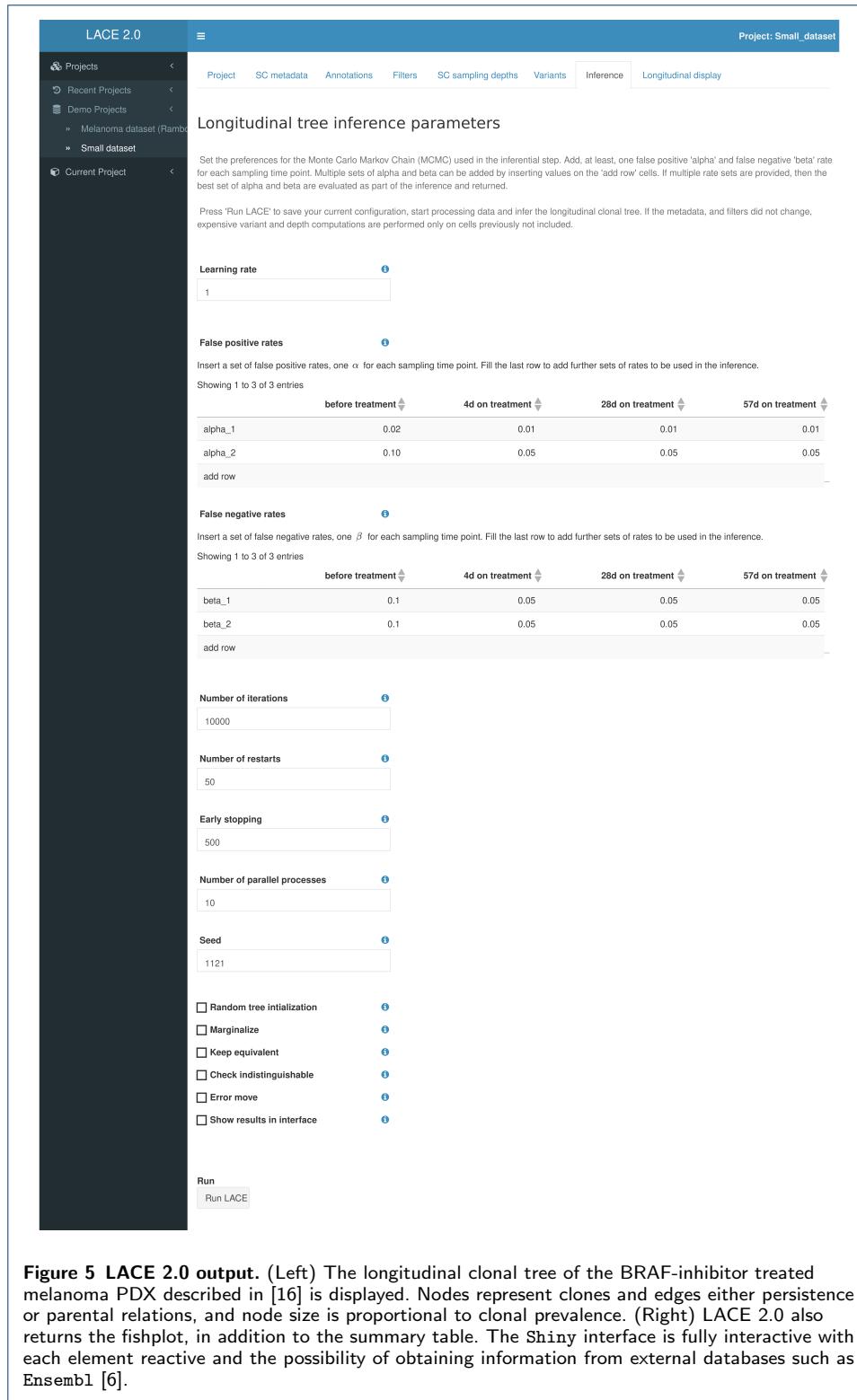
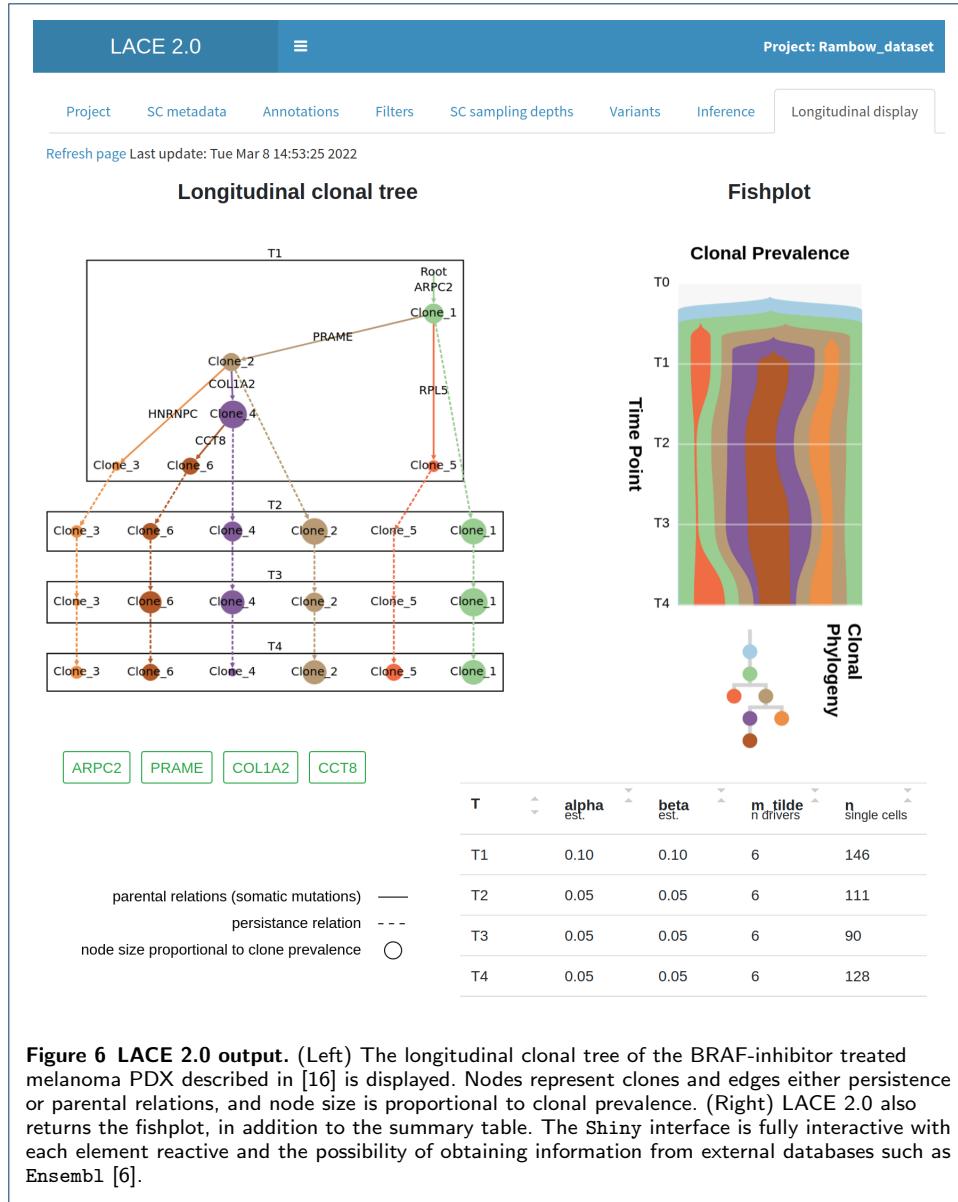


Figure 5 LACE 2.0 output. (Left) The longitudinal clonal tree of the BRAF-inhibitor treated melanoma PDX described in [16] is displayed. Nodes represent clones and edges either persistence or parental relations, and node size is proportional to clonal prevalence. (Right) LACE 2.0 also returns the fishplot, in addition to the summary table. The Shiny interface is fully interactive with each element reactive and the possibility of obtaining information from external databases such as Ensembl [6].

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