LARVA: Large-scale Analysis of Recurrent Variants and Annotations (Eighth Draft)

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Abstract

We present LARVA (Large-scale Analysis of Recurrent Variants and Annotations), a computational framework for studying whole genome sequenced (WGS) DNA of cohorts of genetic disease patients, uncovering mutations and mutated genome annotations that are common to multiple samples. These *recurrent mutations* may correspond to important sites of disruptions for diseases like cancer, and therefore may be crucial for understanding how such diseases progress, and how they may be treated. LARVA may be applied to both somatic variants and rare germline variants, and the presence of both recurrent somatic and germline variants in the same annotation could implicate previously unknown disease-causing variants. In this paper, we explain the concepts of LARVA's framework, and how it functions to identify recurrent mutations and recurrently mutated genome annotations. We illustrate how LARVA may be used to study recurrent mutation patterns in both coding regions and noncoding regulatory elements, and sets of pathways and interaction networks. For the purposes of determining if observed recurrent variation is statistically significant, we introduce a Statistical Assessment Module (LARVA-SAM) to assess the statistical significance relative to recurrent variation expected under neutral mutation processes. Starting with an existing exome model of factors that influence the neutral mutation rate, we have developed our model to simulate expected variation across the entire human genome. Our model makes use of whole genome mutation rate influencers such as DNA replication timing, histone marks, whole genome RNA-seq signals, and SNV density. Our system also provides an Analysis Integration Module (LARVA-AIM) for the integration of multiple LARVA analyses, for deeper understanding of disease variation. We have applied LARVA's methods to sets of prostate cancer WGS data to demonstrate its usefulness.

Introduction

Numerous cancer patients have been genome sequenced (Barbieri 2012, Baca 2013, Grasso 2012), opening up opportunities to identify the underlying genetic causes for cancer phenotypes and develop more effective therapies targeted at specific molecular subtypes of cancer. Most of these studies have been so far focused on identifying mutations and defects in the protein-coding regions, or exomes, of cancerous genomes (Baca 2013). However, this approach ignores investigation of potential variation in important noncoding features of the genome.

There are many noncoding genome regions that influence gene transcription. Such features include pseudogenes, some of which are transcribed and can be incorporated into functional transcripts (Pei 2012). There are also various classes of noncoding RNA, such as microRNA (miRNA), small interfering RNA

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(siRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) that bind and regulate transcripts (Esteller 2011). Furthermore, the binding sites of transcription factors might be affected by cancer, which are important to gene expression levels. Finally, various protein factors can bind to stretches of genomic DNA called enhancers that promote gene transcription.

Two computational systems, HaploReg (Ward 2011) and RegulomeDB (Boyle 2012), were previously developed to determine the effect of GWAS variants on noncoding annotations. HaploReg intersects the variants of WGS samples with a fixed series of noncoding regulatory elements in the human genome, determines the variants' effects on noncoding regulatory motifs, and indicates the chromatin state of the genomic region to which each variant maps. RegulomeDB further develops this idea by expanding the range of genome annotations used to include experimentally verified regulatory regions, ChIP-seq-derived transcription factor (TF) binding sites, eQTL, and DNase footprinting.

Also important to understanding disruptive cancer mutations is the placement of cancer-mutated genes into their systems-level contexts. Identifying the pathways and interactions in which the products of mutated genes participate is often crucial to seeing precisely how cellular functions are being disrupted by cancer (Vandin 2011). Protein interaction networks have also proven useful for characterizing cancer disruption: disrupted subnetworks of interacting proteins have been used to more accurately classify subtypes of breast cancer in Chuang *et al.* (2007)

Recent computational systems that focus on the cancer pathway disruption include cBio (Cerami 2012) and Multi-Dendrix (Leiserson 2013). cBio starts with variant datasets, and a database of genes and their pathway membership information. The cBio system then identifies those pathways mutated with high coverage and high mutual exclusivity. High coverage refers to the presence of mutations in a large proportion of samples, and high exclusivity means that many of the highly damaging, driver mutations appear in mutually exclusive samples, owing to the sufficiency of mutating just one part of a pathway to nullify its function. Multi-Dendrix extends these ideas by introducing new algorithms to find arbitrary sets of genes that exhibit high coverage and mutual exclusivity of variants, rather than being limited to previously established pathways. GEMINI (Paila 2013) is another general system that manages variant call sets and genome annotation sets through an SQL database, and allows users to formulate their own SQL-based queries over the stored data, allowing a wide range of flexibility for exploring variant data.

Here, we present a computational system that supports the study of cohorts of whole genome sequenced (WGS) disease patient samples. The primary function of LARVA (Large-scale Analysis of Recurrent Variants and Annotations) is to identify recurrent patterns of disease mutation in various genome annotations using WGS data from multiple disease patients, and compute the statistical significance of these findings. Our framework makes use of a relational database system approach to organize, maintain, and operate on genome variant and annotation data in a systematic way. LARVA allows users to investigate recurrent

variation patterns that the stored disease variant data present in the stored genome annotation data by casting the relevant questions as SQL queries. This framework accommodates a wide range of query types, spanning any genetic disease, and any set of genome annotations one wishes to study.

On a simple level, a mutation recurrence would be a variant at exactly the same position in two individuals. However, this is exceedingly unlikely for rare variants [PMID: 20981092]. Thus, we will consider mutational burden spread over elements. These elements can be single annotations, such as exons, pseudogenes, noncoding RNA, and regulatory features like promoters and enhancers. On a more complex level, we will consider groups of genes related through a common pathway, or through a protein interaction subnetwork, as a single element, where variants from multiple patients that map anywhere in the gene group represent a recurrence.

Our mutation recurrence discovery procedure has three stages. Given a genetic disease cohort, we will first identify recurrences in the somatic variants. We will then do the same for the rare, germline variants. The third step involves looking for connections between the two sets of recurrent variants: elements that contain recurrent somatic variants and recurrent rare germline variants imply that the elements' overlapping variants may be functionally connected. The absence of common variants from these elements would serve as further evidence for a functional connection to genetic disease.

In addition to recurrent variant identification, LARVA offers two additional modules. Firstly, LARVA includes a Statistical Assessment Module, LARVA-SAM, that uses a model of neutral genome evolution to determine the statistical significance of the recurrent mutation patterns that LARVA identifies. Building on a previously developed null model for exomes (Lawrence 2013), we introduce a null model for whole genomes. Secondly, LARVA's Analysis Integration Module (LARVA-AIM) enables further exploration of a LARVA systems-level analysis. When LARVA is used to study recurrent variation in pathways and networks, LARVA-AIM may be employed to place recurrently mutated genes in their pathway and network context. Recurrent gene and pathway/network data are combined to allow one to observe the number of pathways in which a recurrently mutated gene participates, or the number of network neighbors it has.

We have applied LARVA to cancer data to elucidate patterns of recurrent prostate cancer mutations in important noncoding regulatory features of the genome. LARVA has also been used to explore recurrent mutations on a pathway and interaction network level in this data. The following sections describe LARVA's concepts, and their applications to the study of genetic disease.

LARVA Concepts

One of LARVA's important design features is its use of a ~~relational~~ database to manage its data and express recurrent variant exploration as database queries. We have implemented this using SQLite. The core module provides analysis of

disease variant calls for patterns of recurrent variation in genome annotations. We shall call this module LARVA-Core. This module has two primary inputs: *variant files* and *annotation files*.

The *variant files*, or *vfiles*, are derived from genetic disease patients whose genomes have been sequenced, and for which *single nucleotide variants* (SNVs) have been called by comparing the patients' genomes to a reference genome. Each file corresponds to a single patient's variant calls.

The *annotation files*, or *afiles*, are derived from a number of genome annotation sources. *Afiles* we have collected for LARVA analysis include protein-coding exon, pseudogene, and noncoding RNA data from the GENCODE project (Harrow 2012). We also studied transcription factor binding sites derived from a number of sources (Rozowsky 2009, Kheradpour 2012). Finally, we sought to understand cancer variation on a system-wide level by studying recurrent variation in metabolic pathways and protein interaction networks (Kanehisa 2000, Kanehisa 2011, Prasad 2009).

Measures of Mutation

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LARVA-Core intersects a set of *vfiles* with a set of *afiles* and identifies two types of recurrent mutation. These include:

- *Recurrent variants:* Overlapping SNVs from multiple samples that fall into at least one *afile* annotation (Fig. 1a). Such mutations may correspond to a critical component of the annotation's function that is important for tumor suppression. These mutations may also be used to classify the subtype and severity of cancer patients (Vandin 2011).
- *Recurrently mutated annotations:* Annotations that contain SNVs from multiple samples that do not necessarily overlap (Fig. 1b). Such annotations may be functionally disruptable in multiple places, and therefore, multiple patients with the same functional disruption may carry SNVs in different places of the same gene.

LARVA-Core's findings are presented using three Measures of Mutation. These are computed for each annotation, and each *afile* annotation set. They are:

- *Number of samples mutated*: The number of samples represented by SNVs that fall anywhere in the given annotation, or *afile* annotation set.
- *Number of annotations recurrently mutated*: The number of annotations in an *afile* annotation set that are mutated in at least two samples. Not applicable to individual annotations.
- *Number of recurrent variants*: The number of SNVs from multiple samples that overlap exactly, and fall anywhere in the given annotation, or *afile* annotation set.

LARVA Statistical Assessment Module (LARVA-SAM)

It is important to determine whether the recurrently mutated annotations and recurrent variants of LARVA-Core are statistically significant, in that these patterns are not the result of random, neutral mutation processes. To that end, LARVA has a module for randomly generating sets of cancer variants similar to the actual datasets, and running LARVA-Core on these random datasets to gather information on recurrently mutated annotations and variants that would occur by chance. Hence, a random distribution of the Measures of Mutation is generated, and compared to the actual, observed Measures of Mutation to determine whether the mutation patterns of the actual datasets are statistically significant.

Random variant generation for exome datasets

When LARVA-SAM is used on exome variant datasets, the random variant datasets are derived by simulating the distribution of variants expected for a neutrally evolving exome. Our neutral mutation "null model" is defined as a weight distribution over all genes, where the weight is based on a number of factors that influence their neutral mutation rate (Lawrence 2013). These factors include:

- *Expression level:* More highly expressed genes have higher levels of transcription-coupled repair.
- *DNA replication time:* Early in the DNA replication process, there are more free nucleotides available for DNA repair. As the process continues, this nucleotide pool is depleted, and portions of the genome that are replicated at a late phase are more likely to pick up mutations (Chen 2010).
- *Chromatin state:* Genome regions with open chromatin are less likely to be mutated than regions with closed chromatin (Schuster-Böckler 2012).
- *Length:* Longer genes will pick up more variants by chance than shorter genes.

These factors are used to produce a weight for a gene g using the following function:

$$weight(g) = w_1 log\left(1 - CDF(expression(g))\right) + w_2 log\left(CDF(reptiming(g))\right) + w_3 log\left(1 - CDF(chromatin\_state(g))\right) + w_4 log\left(CDF(length(g))\right)$$

where

- $expression(g)$ is the expression level of gene g, according to the Cancer Cell Line Encyclopedia's (CCLE) RNA-Seq data (Barretina 2012). This is an average of the expression across all CCLE cancers.
- $reptiming(g)$ is the replication timing of gene g, according to Chen *et al.* (2010).
- $chromatin\_state(g)$ is a measure of how open or closed the chromatin is at gene g, according to Lieberman-Aiden *et al.* (2009).
- $length(g)$ is the length of gene g.

- w_1 … w_4 are the weights assigned to each variable to represent differing contribution levels.

CDF here refers to the cumulative distribution function of the expression values, replication timing values, etc. It functions as a percentile ranking of each variable within its respective distribution, and influences *weight*(*g*) accordingly. For example, genes with higher expression are less likely to be mutated due to transcription-coupled repair, therefore lower expression values will map to higher weights, and higher expression values will map to lower weights. Hence, the more likely a gene is to be mutated, the higher its weight.

Once the gene to place the random variant in has been chosen, the gene's exon coordinates are retrieved, and an exact position for the random variant is determined by selecting one at random from the retrieved exons, with uniform probability. This procedure is repeated for each variant to be generated for the given random variant file.

LARVA-SAM will generate a user-specified number, *nrand*, of replicates of the *vfiles* dataset that represents the actual data. Each of these replicate datasets contain the same number of *vfiles* and variants as the original dataset, but have randomized variant positions.

Random variant generation for whole genome datasets

When LARVA-SAM is used on whole genome variant datasets, LARVA's whole genome neutral mutation null model is used. Our whole genome weight function assigns weight to discrete partitions of the entire genome, rather than genes. The factors used in our whole genome model include:

- *DNA replication time:* As in the exome model, later replicating portions of the genome are more likely to pick up mutations (Chen 2010).
- *H3K4me1 and H3K4me3 marks:* Schuster-Böckler and Lehner (2012) demonstrated that H3K4me1/me3 marks are anti-correlated with SNV density.
- *Expression level:* As in the exome model, more highly expressed genome regions have higher levels of transcription-coupled repair.
- *SNV density:* Studies of population variation from the 1000 Genomes Project have indicated which regions are predisposed to variant accumulation (Durbin 2010).

The weight of a region *r* in the genome is defined with the following function:

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$$weight(r) = w_1 log\left(CDF\left(reptiming(r)\right)\right) + w_2 log\left(1 - CDF\left(H3K4me1(r)\right)\right) + w_3 log\left(1 - CDF\left(H3K4me3(r)\right)\right) + w_4 log\left(1 - CDF\left(expression(r)\right)\right) + w_5 log\left(CDF\left(SNV\_density(r)\right)\right)$$

where

- r is a 100,000-bp-long block of the human genome (hg19 build).
- $reptiming(r)$ is the replication timing of region r, according to Chen *et al.* (2010)
- $H3K4me1(r)$ is the level of histone H3K4 mono-methylation of region r, according to ENCODE GM12878 Peak-seq experiments (Dunham 2012).
- $H3K4me3(r)$ is the level of histone H3K4 tri-methylation of region r, according to ENCODE GM12878 Peak-seq experiments (Dunham 2012).
- $expression(r)$ is the expression level of region r, according to the ENCODE's GM12878 RNA-seq track (Dunham 2012).
- $SNV\_density(r)$ is the number of SNVs in region r, according to the 1000 Genomes Project (Durbin 2010).
- $w_1 \ldots w_5$ are the weights assigned to each variable to represent differing contribution levels. These can be adjusted to fit the model to the observed contributions of each factor.

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Additional whole genome signals that influence genome-wide mutation rates will be iteratively incorporated in followup work. As with the exome null model, the exact variant position is determined by randomly choosing a position in the selected region with uniform probability. This whole genome method of random variant placement represents an extension of Lawrence *et al.'s* (2013) methods to account for the systemic biases in effect on the human genome's neutral mutation rate.

LARVA-Core runs and Normal distribution fitting

After the random variant generation step, LARVA-SAM will have generated *nrand* random variant datasets. These datasets are used as input for LARVA-Core, generating *nrand* datapoints approximating the expected distribution of each Measure of Mutation. These datapoints are fit to a Normal distribution, and compared to the corresponding Measure of Mutation from the actual *vfile* data to produce a *p*-value, for significance testing.

LARVA Analysis Integration Module (LARVA-AIM)

LARVA-Core may be used for numerous types of analyses, the results of which can be integrated for better understanding of disease variation. To this end, we have developed the LARVA Analysis Integration Module (LARVA-AIM), designed to facilitate the integration of multiple analyses after significance testing. Our collaborating cancer researchers find this module's features very helpful.

To assist in the systems-level analysis of disease variant files, LARVA-AIM may be used to integrate a LARVA-Core gene analysis and a LARVA-Core pathway analysis. LARVA-AIM can take a list of recurrently mutated genes and place them in the pathways in which those genes participate. Additionally, LARVA-AIM can be used to understand recurrently mutated genes in the context of their protein products' interactions. The AIM module can bring recurrently mutated gene analysis data and protein interaction network data together, so users can see the number of interaction partners for each recurrently mutated gene. This enables the identification of potential disease-related network hubs.

Example Workflows of applications using LARVA

By plugging a genetic disease cohort's variant calls into LARVA's *vfiles* parameter, and using different settings of LARVA's *afiles* parameter, one may use LARVA to study a cancer cohort's patterns of recurrent variation over many genome annotations of interest. We illustrate this flexibility with the following examples.

1) ***afiles* = noncoding RNA annotations.** With this setting, LARVA can find potential regulatory drivers from a genetic disease cohort in noncoding RNA (ncRNA). ncRNA annotations may be derived from the GENCODE project (Harrow 2012). Recurrent variants corresponding to putative critical point mutations in ncRNA will be identified, as well as any ncRNA mutated in multiple samples.
2) ***afiles* = KEGG pathways.** Here, one may define an *afile* for each pathway in the KEGG database (Kanehisa 2000, 2011), each containing the pathway members. Under this arrangement, one may study a genetic disease cohort's recurrently mutated pathways using LARVA's annotation set Measures of Mutation. Once pathways worth closer investigation are identified at this higher level of analysis, one may drill down into the annotation Measures of Mutation for those pathways to investigate further.
3) ***afiles* = Transcription factor binding peaks.** Using data on the binding sites of transcription factors from ENCODE Peak-seq experiments (Rozowsky 2009), one may use LARVA to identify recurrent mutations that may lead to expression dysregulation in a genetic disease cohort. By defining an *afile* for each transcription factor, each containing that factor's sites, one may identify both factors and sites that should be studied further.

LARVA Applications to Cancer

We have applied LARVA to studying recurrent variants and recurrently mutated annotations in a number of prostate cancer datasets (Berger 2012, Weischenfeldt 2013, Barbieri 2012, Baca 2013). Our findings have produced new insights into potential noncoding disruptions in these cancers.

LARVA's source code is available to download through Github, at <https://github.com/gersteinlab/larva>, and is also available as a web server at <larva.gersteinlab.org>. Each has their advantages and target audiences.

The Github version is best suited for a large analysis on dedicated servers. The target audience for this version is experienced bioinformaticians knowledgeable in installing the required dependencies, and with access to large servers.

On the LARVA website, users may upload variant and annotation datasets for LARVA to work with, without having to configure a local installation of LARVA. A multi-file selector is available to upload all the relevant data files in a single step, after which the user is given the option to run LARVA-Core, or any of the other LARVA modules described above. The website version of LARVA is best suited for a small, quick analysis. The target audience for this version is translational researchers or clinicians with little command line experience, and without access to large servers.

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Discussion

In this paper, we have introduced a new computational framework for exploring patterns of recurrent mutation across cohorts of patients with genetic disease. LARVA is designed to be used to explore a broad range of genome annotations to uncover the ones that are mutated across many samples, making it possible to predict putative drivers of genetic disease, and prioritize these predicted drivers for more rigorous downstream analysis. This may lead to faster identification of important targets that may be used to suppress disease in therapies and drugs.

Using a relational database design, LARVA is easily adaptable to many different types of analyses. It may be used to study recurrent mutation patterns across genes, pseudogenes, noncoding RNA, and various noncoding regulatory elements. This ability to study noncoding mutation serves as an important supplement to the many exome-focused studies that have been conducted so far on genetic diseases, such as cancer. LARVA may also be used to study genetic diseases at a systems level, with analyses on pathways and interaction networks possible.

Furthermore, we have developed LARVA-SAM, a module designed to compare observed variant file recurrent mutation patterns to a simulated distribution of variants generated from a neutrally evolving genome model. This comparison allows users to identify genome annotations that are mutated in a higher number of samples, or have a higher number of recurrent variants, than expected under neutral evolution, indicating possible cancer involvement. Finally, we have created LARVA-AIM, a module with the purpose of bringing together recurrent mutation data from multiple types of analyses to shed deeper understanding on features with probable connections to cancer disruption processes.

LARVA Computational Efficiency and Parallelization

Due to the large number of simulated LARVA-Core runs that LARVA-SAM executes, LARVA-SAM is very compute intensive. Therefore, we have developed a parallel version of LARVA-SAM that leverages multi-core CPUs. Users may specify the number of CPU cores on their machines that LARVA should use. LARVA-SAM will automatically split its LARVA-Core runs across the specified

number of cores evenly, and process each batch in parallel. This allows the system to run as efficiently as the available hardware allows. We have shown that this parallel implementation greatly speeds up the running time for a large number of LARVA-Core runs.

Future Work

In addition to recurrence information, functional annotation is important to assessing a variant's likelihood of disease association (Khurana 2013). In the future, we plan to add functional annotation capabilities to LARVA, enabling the filtering of results for recurrent variants and recurrently mutated annotations more relevant to diseases. We will also continue to improve LARVA's algorithms and LARVA's user interface. As the amount of genetic data increases, it will be important to further optimize LARVA's computational efficiency, and therefore we are investigating these issues for future iterations of LARVA. Also, we will continue to gain insights by applying LARVA to additional cancer types and subtypes. In the long term, we envision LARVA becoming increasingly useful for elucidating important insights and understanding about all types of genetic diseases.

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