

# **Gene Identification via Phenotype Sequencing**

**Version 1.6**

**User manual**

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# Chapter 1. Algorithm

## 1.1 Overview

The advent of next generation sequencing has enabled fast and cost-effective genotyping, which may significantly accelerate the process of gene identification in forward genetics studies. Sequencing-based forward genetics studies are frequently referred to as phenotype-sequencing studies.

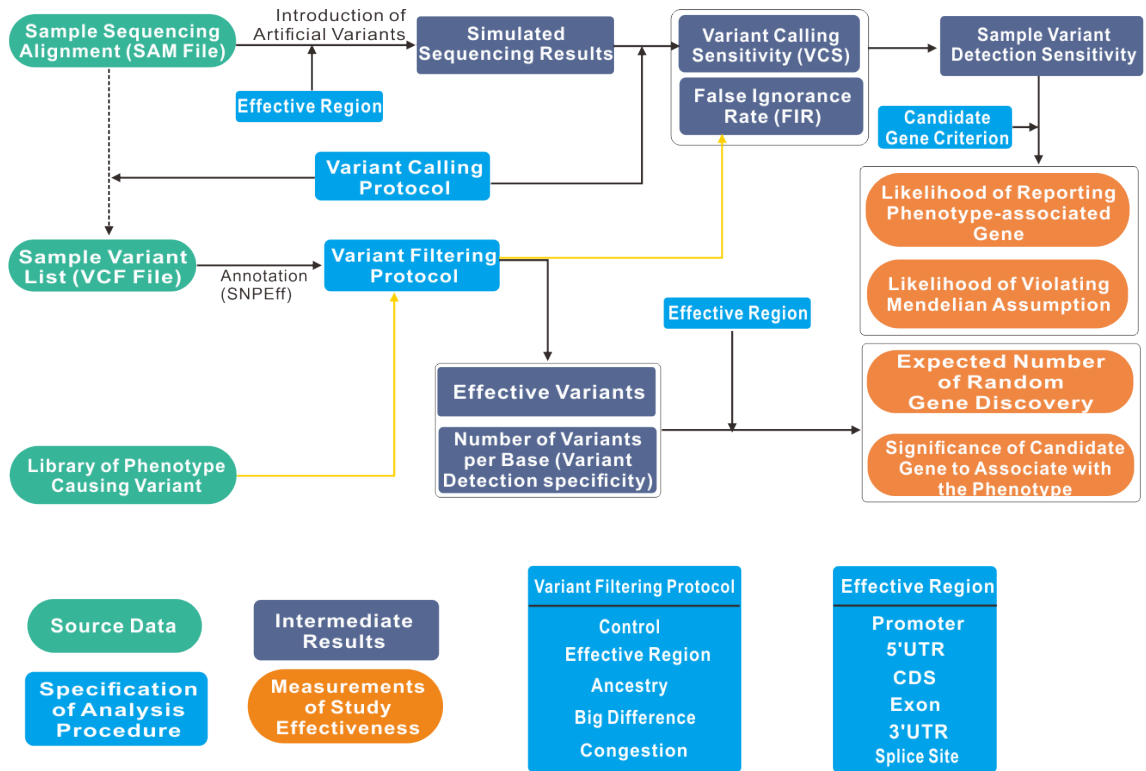
The success of a typical phenotype-sequencing study depends on a range of experimental and analysis choices, including the number of phenotype-exhibiting samples being sequenced, the genomic region being sequenced, the quality and depth distribution of sequencing data, the approach to map sequencing reads to genome, the variant calling method, the approach to filter unlikely-causal variants, and the criterion to report candidate genes. Some previous studies have characterized the impacts of some of these choices (Ratan, Miller et al. 2013, Chilamakuri, Lorenz et al. 2014, Lelieveld, Spielmann et al. 2015). However, for investigators, it is still difficult to design an optimal analysis procedure with an integral consideration of all factors that affect its chance of success. In particular, after the sequencing results are ready, how to design an effective analysis procedure that fits the quality of this particular set of sequencing data.

To meet this analytical need, we developed the Gene Identification via Phenotype Sequencing (GIPS) tool. GIPS estimates four measurements to help optimize an analysis procedure. The four measurements are, **1) its chance to report candidate genes that are truly associated with the phenotype; 2) the expected number of random genes that may be reported; 3) the significance of each candidate gene to associate with the phenotype; and 4) the significance of violating Mendelian assumption, if no gene is reported or all candidate genes have failed validation.** Their interpretations are discussed in more detail in section 3.2.4 (Example discussion: strategy to optimize an analysis procedure).

As previously described, a range of experimental and analysis parameters can affect these four measurements of study effectiveness. In general, their impacts on these four study-wise effectiveness measurements can be summarized into their impacts on two sample-wise effectiveness measurements: the “sensitivity” and “specificity” for each sample, with which an analysis procedure under evaluation is expected to detect the phenotype-causing variants. Note that in this framework, it is permitted that different analysis procedures can be applied on different samples, which allows fine-tuning of sample-specific analysis procedures based on sample-specific data qualities. With the sample-wise variant detection sensitivities and specificities, the combined study-wise measurements of analysis effectiveness can be computed in a recursive form, which is detailed in later sections.

Figure 1 illustrates the general workflow of GIPS, which formally considers seven aspects of an

analysis procedure that affect its likelihoods of success in gene identification. These aspects include, 1) the number of phenotype-exhibiting samples being sequenced, 2) the genomic region being sequenced, 3) the quality and depth distribution of sequencing data, 4) the choice of software and parameters to align sequencing reads, 5) the choice of software and parameters to call variants, 6) the choice of strategies to filter variants that are unlikely to associate with the phenotype, and 7) the criterion to report candidate genes. The impacts of these choices can be estimated from real data, or customarily specified to reflect the belief of the investigator.



**Figure 1.** The general workflow of GIPS. Details are given in later sections.

In general, impacts of “the genomic region being sequenced” and “the approach to filter unlikely-causal variants”, on the variant detection sensitivity of each sample, can be estimated by using the same approach to filter a library of known phenotype-causing variants, and compute how much proportion of the phenotype-causing variants will be discarded. For human, the ClinVar database collected 19,334 Mendelian phenotype-associated variants (Landrum, Lee et al. 2014). Because the genome structures in higher organisms are similar, ClinVar may also be used as a reference library for other higher organisms, in case no appropriate library exists.

Impacts of “the quality and depth distribution of sequencing data”, “the approach to map sequencing reads to genome”, and “the variant calling method”, on the variant detection sensitivity of each sample, can be estimated by simulating a set of sequencing data with the same quality and depth



distribution. The simulated sequencing reads are from a genome containing random artificial (simulated) variants. Therefore, the combined impact of these factors on variant detection sensitivity can be estimated through computing how much proportion of these artificial SNPs can be detected.

GIPS measures the sample-wise specificities of an analysis procedure by computing the frequency of detected variants per base in the effective genomic region (after all variant filtering steps). Assuming that all detected variants are unrelated to the phenotype, these frequencies are used to compute how many genes are expected by chance to accumulate random mutations in multiple samples and pass the candidate gene criterion.

## 1.2 Variant detection sensitivity

Variant detection sensitivity (VDS) is computed for a specific analysis procedure that is applied on a specific sample. It has two components, variant calling sensitivity and false ignorance rate.

Variant calling sensitivity (VCS) characterizes how much proportion of the real variants can be observed with the analysis procedure under evaluation, before any filtering. It depends on the quality and depth distribution of the sequencing data, the approach to align sequencing reads, and the approach to call variants.

It is not expected all variants are phenotype-related, e.g., synonymous mutations are unlikely to produce phenotype. In most phenotype-sequencing studies, filters are applied to remove observed variants that are unlikely to produce the target phenotype. In GIPS, false ignorance rate (FIR) is used to characterize the likelihood of filters to discard variants that may actually produce the phenotype.

Focusing on an “effective genomic region” (e.g., using exome sequencing) is considered a filter, because this action risks ignoring phenotype-associated variants that are located outside the effective genomic region. In addition, GIPS supports the use of four types of other filters. They are, **1) the control filter, which removes variants observed in phenotype-absent control samples; 2) the ancestry filter, which removes variants replicated in multiple phenotype-exhibiting samples (this filter is applicable only if all samples are known unrelated); 3) the big difference filter, which removes small-difference non-synonymous mutations that are unlikely to change protein function; and 4) the congestion filter, which removes potentially spurious variants that are results of ambiguous alignments of sequencing reads.**

Let:

$D_{S_i}$  be the VDS of sample  $S_i$ ;

$H_{S_i}$  be the VCS of sample  $S_i$ ;

$\Phi_{S_i}$  be the FIR of sample  $S_i$ ;

We have:

$$D_{S_i} = H_{S_i} \times (1 - \Phi_{S_i}).$$

For each sample  $S_i$ ,  $H_{S_i}$  and  $\Phi_{S_i}$  can be estimated from real data or customarily specified. Note that GIPS allows the usage of sample-specific sequence alignment/variant calling procedures.

### 1.2.1 Variant calling sensitivity

The VCS  $H_{S_i}$  of sample  $S_i$  can be estimated with real sequencing data. To estimate  $H_{S_i}$  for homozygous variants, the raw sequencing reads from sample  $S_i$  are first mapped to the reference genome. GIPS will then simulate an alignment result from a hypothetical genome of sample  $S_i$ , which harbors artificial SNPs. A number (the “*NUM\_SIM\_SNPS*” parameter in “*PROJECT.ini*”, defaults to 5,000) of artificial SNPs, distributed uniformly over the “effective genomic region”, are hypothetically introduced to the genome of  $S_i$ . The aligned sequencing reads are updated to reflect the introduction of artificial SNPs. To avoid complications, the artificial SNPs are only introduced at locations where the genome of  $S_i$  matches the reference genome. The artificial SNPs randomly change the original nucleotide bases to other ones. The mapped reads in the original alignment result (SAM file) are updated to reflect this change. For each mapped read covering an artificial SNP, its corresponding base is changed as follows. If the base is a correct read (i.e., matching the reference genome), it is changed to match the artificial SNP. If the base is an incorrect read and it does not match the artificial SNP, it is left unchanged. If the base is an incorrect read and it matches the artificial SNP, it is changed to match the reference genome. After this update, the simulated mapping result and the original mapping result will have the same reads quality, mapping quality, and depth distribution. The VCS  $H_{S_i}$  of sample  $S_i$  is therefore estimated as the proportion of these artificial SNPs that can be detected with the user-supplied variant calling procedure (script).

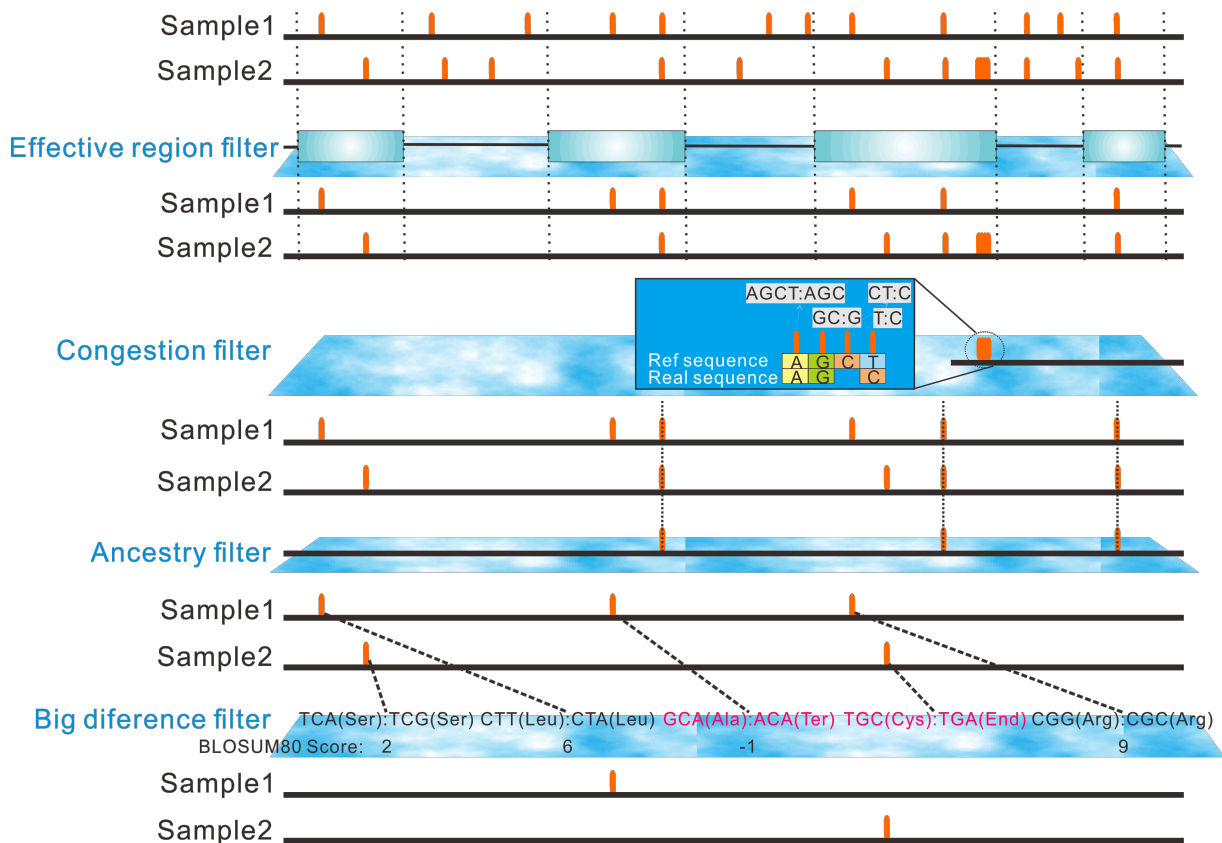
Users may specify any variant calling protocol with a custom script, as long as it takes a sequence alignment result in SAM format (Li, Handsaker et al. 2009) as input and produces a variant call file in VCF format. Because GIPS will invoke the user-supplied variant-calling scripts to call variants from the simulated alignment results, these scripts have to implement a convention of command line arguments. See 2.2.2 for details. Note that GIPS will not invoke these scripts to call variants from the original alignment results. Instead, users should use their scripts to call variants from the original alignment results and supply the resultant VCF files to GIPS. This design is also to ensure that the user-supplied variant calling scripts are working.

To estimate  $H_{S_i}$  for heterozygous variants, GIPS uses the same approach except that only a random half of the mapped reads in the original sequence alignment result (SAM file) are updated to

reflect the introduction of artificial SNPs.

### 1.2.2 False ignorance rate

The FIR  $\Phi_{S_i}$  of sample  $S_i$  does not depend on the sequencing result of  $S_i$ . It depends only on the variant filters that are applied on  $S_i$ . The same filtering procedure will always have the same FIR, however, it is allowed to use different filtering procedures on different samples. GIPS supports the use of five configurable filters (Figure 2). Two filters, the effective region filter and the big difference filter, are based on predicted variant functions (i.e., variant annotations), which aim to discard variants that are unlikely to produce any phenotype. The other three filters, the ancestry filter, the control filter, and the congestion filter, are study-based filters, which aim to discard variants that are unlikely to produce the target phenotype. Details about these filters are given below.



**Figure 2.** Variant filters.

Let:

$\Phi_{S_i}$  be the FIR of sample  $S_i$ ;

$\Phi_{S_i,F}$  be the FIR of function based variant filters that are applied on  $S_i$ ;

$\Phi_{S_i,S}$  be the FIR of study based variant filters that are applied on  $S_i$ ;

We have:

$$\Phi_{S_i} = 1 - (1 - \Phi_{S_i,F}) \times (1 - \Phi_{S_i,S}).$$

The FIR of function-based variant filters  $\Phi_{S_i,F}$  is estimated by applying the same filters on a library of known phenotype-causing variants, and compute how much proportion of these variants will be discarded. For human, the ClinVar database lists 19,334 Mendelian phenotype-associated variants (Landrum, Lee et al. 2014). For other higher species, because the genome structures in higher organisms are similar, ClinVar may also be used as a reference library in case no appropriate library for these species exists. Users can specify the reference variant library with the “*LIB\_PHENOTYPE\_VAR*” parameter in “*PROJECT.ini*”. The ClinVar database has been included in the GIPS distribution package. If no reference variant library is specified, ClinVar will be used as default.

The FIR of study based variant filters  $\Phi_{S_i,S}$  is estimated as the proportion of “effective genomic region” that is ignored by these filters. Study based variant filters discard variants according to variants detected in other samples. However, variants detected in other samples could be spurious. Therefore, GIPS takes the proportion of “effective genomic region” ignored by these study based variant filters as their estimated FIR.

Whether to use a specific variant filter is specified with the “*VAR\_FILTERS*” parameter in the “*[GLOBAL]*” section of the parameter file “*PROJECT.ini*”.

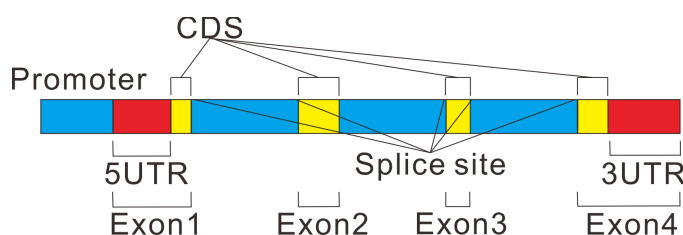
*VAR\_FILTERS*: [EBAC]

E: Effective genomic region; B: Big difference filter; A: Ancestry filter; C: Congestion filter.

Note that the “effect genomic region” filter is required. It will take effect even if “E” is not specified here. In addition, the “control filter” will take effect if control samples are specified. See 1.2.2.5 for details. No specification of control filter is needed with this parameter. “C” here stands for congestion filter.

### 1.2.2.1 Effective region filter

This filter discards variants that are outside the user-specified “effective genomic region”. Effective genomic region is specified based on the reference genome structure. An annotation of the reference genome is needed in generic feature format (GFF3) (Stein 2007). GIPS uses the below gene model to specify the effective genomic region (Figure 3).



**Figure 3.** Gene structure model for specification of effective genomic region.

Users can specify whether to include the promoter region (keyword: *Promoter=length*), 5' untranslated region (keyword: *5UTR*), coding sequence (keyword: *CDS*), splice site (keyword: *SpliceSite=length*), exon (keyword: *EXON*), and 3' untranslated region (keyword: *3UTR*) into the effective genomic region. The lengths of promoter and splice site can be customized. The effective region specification applies to all samples. The “[GLOBAL]” section of the parameter file “*PROJECT.ini*” contains this specification:

*REF\_GENOME\_ANNOTATION.GFF*: /path/to/genome/annotation/file.gff3

*EFF\_REGION*: Keywords separated by “|”. Defaults to “CDS|SpliceSite=2”.

Example:

*EFF\_REGION*: Promoter=1000|5UTR|CDS|3UTR|SpliceSite=2

### 1.2.2.2 Big difference filter

This filter discards non-synonymous variants that result in similar amino acids. The variants observed in each sample are first annotated by SnpEff (Cingolani, Platts et al. 2012). SnpEff assigns variants into different categories based on their putative effects. If this filter is used, GIPS will keep only variants that are annotated with “*HIGH*” effect and coding region variants that lead to significant amino acid changes. To define what is “significant amino acid change”, users can specify a maximum

residue similarity score and an amino acid scoring matrix. GIPS has 87 built-in scoring matrices, which are retrieved from NCBI (<ftp://ftp.ncbi.nih.gov/blast/matrices>).

Big difference filter can have sample specific parameters. The parameters “*SCORE\_MATRIX*” and “*MAX\_AA\_SCORE*” specified in the “[*GLOBAL*]” section of “*PROJECT.ini*” apply to all samples. The “*SCORE\_MATRIX*” and “*MAX\_AA\_SCORE*” parameters can also be specified in specific sample sections, which override the corresponding global parameters.

*SCORE\_MATRIX*: Name of a scoring matrix, e.g., BLOSUM80. Defaults to “DEFAULT”, which discards only synonymous SNPs. Available scoring matrices are listed in the “*config*” file (“*/path/to/GIPS\_folder/config*”).

*MAX\_AA\_SCORE*: Maximum amino acid similarity score to define a significant amino acid change, as in the specified scoring matrix. Defaults to 0.

### 1.2.2.3 Ancestry filter

This filter discards variants that are detected in more than one phenotype-exhibiting samples. If all phenotype-exhibiting samples are known unrelated, the event of random mutations hitting the same genomic position in two or more samples is unlikely. Therefore, the same variant observed in unrelated samples probably indicates a shared genetic background, rather than phenotype association.

Note that the ancestry filter should only be used when all the phenotype-exhibiting samples are known unrelated. For example, in plant research, mutants generated by chemical treatments are unrelated. The next version of GIPS will support gene identification with family-based experimental design.

As described above, the FIR of the ancestry filter is estimated as the proportion of “effective genomic region” covered by all detected variants in all phenotype-exhibiting samples. This estimation implements the consideration that mutations in two unrelated samples do have a small chance of hitting the same genomic location, and that some of the observed variants are spurious.

### 1.2.2.4 Congestion filter

Some genomic regions are highly similar, which makes alignment of sequencing reads difficult. Variants in these regions further complicate the alignment process. As a result, variants observed in these genomic regions are more frequently false positive calls, especially in cases when many variants appear in a short region. Congestion filter discards a variant if there is another variant exists in its vicinity (11bp region centering on it).

As described above, the FIR of congestion filter is estimated as the proportion of “effective genome region” covered by all discarded variants and their 11bp vicinity regions.

#### 1.2.2.5 Control filter

This filter discards variants observed in phenotype-absent control samples. The FIR of control filter is estimated as the proportion of “effective genomic region” covered by all observed variants in all control samples. This estimation implements the consideration that some of the observed variants in control samples are spurious.

The “*CONTROL*” parameter specified in the “[*GLOBAL*]” section of “*PROJECT.ini*” applies to all phenotype-exhibiting samples. The “*CONTROL*” parameters specified in specific sample sections override the global parameter for the respective samples.

Control variants are provided with one VCF file.

*CONTROL*: /path/to/control\_sample.vcf

If there are multiple control samples, please merge the variants called from all control samples into one VCF file.

### 1.3 Variant detection specificity

GIPS measures the specificity of an analysis procedure by computing the frequency of detected variants per base (after all variant filtering steps) in the effective genomic region. This frequency is termed background variant frequency (BVF) .

Let:

$B_{S_i}$  be the BVF of sample  $S_i$ ;

$Nv_{S_i}$  be the number of variants detected (after filtering) from sample  $S_i$ ;

$l$  be the length of the effective genomic region;

We have:

$$B_{S_i} = Nv_{S_i} / l .$$

### 1.4 Measurements of study effectiveness

To report a candidate gene, the criterion is specified as a minimal frequency with which the

candidate gene harbors variants that are detected (after filtering) in phenotype-exhibiting samples.

Let:

$N$  be the total number of phenotype-exhibiting samples;

$M$  be a user specified threshold.

The reporting criterion is therefore:

In  $\geq M$  out of  $N$  phenotype-exhibiting samples, the candidate gene harbors detected variants (after filtering).

With this candidate gene criterion, GIPS calculates four measurements of study effectiveness, which are detailed below.

#### 1.4.1 Significance of each candidate gene to associate with the phenotype

The significance of a candidate gene to associate with the phenotype is calculated as the likelihood with which this candidate gene might accumulate random variants in  $\geq M$  phenotype-exhibiting samples by chance.

Let:

$G_j$  be the candidate gene;

$L_{G_j}$  be the length of effective genomic region that is annotated to  $G_j$ ;

$B_{S_i}$  be the BVF of sample  $S_i$ .  $B_{S_i}$  is obtained earlier.

$PV_{G_j, S_i}$  be the probability of  $G_j$  harboring at least one random variant in sample  $S_i$ ;

We have:

$$PV_{G_j, S_i} = 1 - (1 - B_{S_i})^{L_{G_j}}.$$

Let:

$u, v$  be variables in the recursive function;

$R_{G_j, u}$  be the number of samples from the first  $u$  samples in which  $G_j$  harbors at least one random variant;

We have:



$$\begin{aligned}
& P(R_{G_j, u} = v) \\
&= \begin{cases} PV_{G_j, S_u} \times P(R_{G_j, u-1} = v-1) + (1 - PV_{G_j, S_u}) \times P(R_{G_j, u-1} = v) & (0 < v < u) \\
\prod_{i=1}^u [1 - PV_{G_j, S_u}] & (v = 0) \\
\prod_{i=1}^u PV_{G_j, S_u} & (v = u)
\end{cases}
\end{aligned}$$

The above recursive function enumerates all possible combinations of  $v$  samples from the first  $u$  samples, and sums up the probability of each combination to have  $G_j$  harboring at least one random variant in each sample.

Therefore, the likelihood with which  $G_j$  accumulates random variants in  $\geq M$  samples from all  $N$  samples can be computed as follows.

Let:

$Q_{G_j}$  be the significance of gene  $G_j$  to associate with the phenotype;

We have:

$$Q_{G_j} = P(R_{G_j, N} \geq M) = \sum_{k=M}^N P(R_{G_j, N} = k).$$

#### 1.4.2 Expected number of random genes that may be reported

The likelihood with which a gene can accumulate random variants in  $\geq M$  phenotype-exhibiting samples,  $P(R_{G_j, N} \geq M)$ , is calculated in the previous section. Therefore, the number of random genes that are expected to meet the reporting criterion can be calculated as the sum of this likelihood for all genes.

Let:

$E(M, N)$  be the number of random genes that are expected to meet the reporting criterion;

We have:

$$E(M, N) = \sum_{j \in [all \ genes]} P(R_{G_j, N} \geq M) = \sum_{j \in [all \ genes]} Q_{G_j}.$$

#### 1.4.3 Chance to report candidate genes that are truly associated with the phenotype

The chance that the true phenotype-associated gene will meet the candidate gene criterion is computed as follows.

Let:

$u, v$  be variables in the recursive function;

$D_{S_i}$  be the VDS of sample  $S_i$ .  $D_{S_i}$  is obtained earlier.

$A_u$  be the number of samples from the first  $u$  samples in which the true phenotype-associated variants are detected;

We have:

$$P(A_u = v) = \begin{cases} D_{S_u} \times P(A_{u-1} = v - 1) + (1 - D_{S_u}) \times P(A_{u-1} = v) & (0 < v < u) \\ \prod_{i=1}^u (1 - D_{S_i}) & (v = 0) \\ \prod_{i=1}^u D_{S_i} & (v = u) \end{cases}$$

The above recursive function enumerates all possible combinations of  $v$  samples from the first  $u$  samples, and sums up the probability of each combination to have the true phenotype-associated variants detected in each sample.

Let:

$H(M, N)$  be the chance of reporting the true phenotype-associated gene;

We have:

$$H(M, N) = P(A_N \geq M) = \sum_{k=M}^N P(A_N = k).$$

#### 1.4.4 Significance of violating Mendelian assumption

The significance of violating Mendelian assumption, if no gene is reported or all candidate genes have failed validation, is the likelihood with which true phenotype-associated variants are not detected in  $\geq M$  phenotype-exhibiting samples.

Let:

$w'$  be the number of samples in which the true phenotype-associated variants are detected.

$$w' < M.$$

$w$  be the maximum number of samples in which one gene harbors variants in all these samples, and, this gene has not been proven unrelated to the phenotype.  $w' \leq w$ .

$Z(w', N)$  be the significance of violating Mendelian assumption.

$u, v$  be variables in the recursive function;

$D_{S_i}$  be the VDS of sample  $S_i$ .  $D_{S_i}$  is obtained earlier.

$A_N$  be the number of samples from all  $N$  samples in which the true phenotype-associated

variants are detected.

We have:

$$P(A_u = v) = \begin{cases} D_{S_u} \times P(A_{u-1} = v - 1) + (1 - D_{S_u}) \times P(A_{u-1} = v) & (0 < v < u) \\ \prod_{i=1}^u (1 - D_{S_u}) & (v = 0) \\ \prod_{i=1}^u D_{S_u} & (v = u) \end{cases}$$

Because  $w'$  is difficult to obtain, we compute  $Z(w, N)$  as the upper bound of  $Z(w', N)$ .

Let:

$Z(w, N)$  be the significance of violating Mendelian assumption;

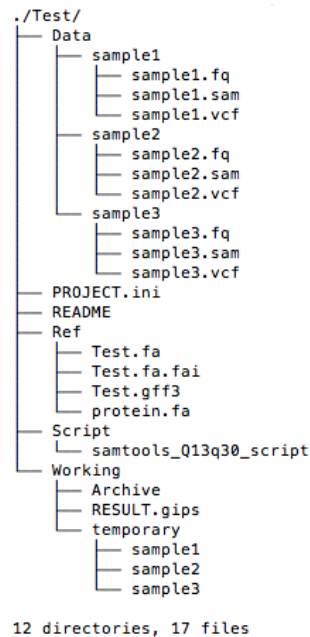
We have:

$$Z(w', N) \leq Z(w, N) = P(A_N \leq w) = \sum_{k=0}^w P(A_N = k).$$

## Chapter 2. Tool Usage

### 2.1 General workflow

To initiate a project, GIPS will create a main directory with four sub-folders. Using the “*Test*” project as example (detailed in 3.1), the structure of project directory is illustrated below (Figure 4). The GIPS parameter file “*PROJECT.ini*” will be placed in the main project folder, and the GIPS result file “*RESULT.gips*” will be placed in the “*Working*” sub-folder.



**Figure 4.** Structure of the GIPS project directory for “test” project.

“*PROJECT.ini*” specifies the analysis procedure that will be evaluated by GIPS. A full analysis procedure includes steps to align sequencing reads to reference genome, to call variants from the aligned reads, to filter the observed variants, and finally, to call candidate genes.

Users need to create custom scripts to implement their desired approach to align sequencing reads and to call variants. These scripts are recommended to be placed in the “/path/to/project\_folder/Script” folder. In addition, it is recommended to use a naming convention that intuitively states the nature of these scripts, for example, “mapper-v1-20150502-q30-nr.sh”. Note that GIPS will not directly invoke user scripts to align sequencing reads or to call variants from the actual sequencing results. GIPS only uses these scripts to process simulated reads to estimate the variant calling sensitivity for each sample (see 1.2.1 for details). Instead, users should use these scripts to call variants from the actual sequencing results, and supply the resultant VCF files to GIPS. Furthermore, it is recommended to use a naming convention for all intermediate result files that unambiguously associates a result file to its producing script. For example, the alignment result of sample\_1 produced by “mapper-v1-0515-q30-nr.sh” is

named “sample\_1-mapper-v1-0515-q30-nr.sam”. The “/path/to/project\_folder/Data” folder is designed to store intermediate result files.

To filter variants that are unlikely to associate with the phenotype, users can specify a filtering procedure with five configurable filters (see section 1.2.2 for details). Finally, the candidate gene criterion is specified.

After an analysis procedure has been fully specified in “*PROJECT.ini*”, a GIPS run will execute and evaluate this procedure. Candidate genes will be reported and the four measurements of study effectiveness will be computed. The “*RESULT.gips*” file reports the GIPS results. A summary of the analysis procedure and intermediate results is also included in “*RESULT.gips*”.

After the first GIPS run, users can iteratively optimize the analysis protocol and evaluate how these changes affect candidate genes and study effectiveness. Users can modify “*PROJECT.ini*” and run GIPS in the “update” mode. In the “update mode”, GIPS will try to reuse previously produced intermediate results whenever possible. Meta data on previous intermediate results are coded and stored in the “[*GIPS TRACEBACK*]” section of the “*RESULT.gips*” file. In each GIPS run, the existing “*RESULT.gips*” file, which contains information of the previous GIPS run, will automatically be archived in the “/path/to/project\_folder/Working/Archive” folder. If a user wishes to base further analysis optimization on an earlier analysis procedure (not the procedure of the immediate previous run), he can locate the corresponding “*RESULT.gips*” file in the archive folder and restore this file to “/path/to/project\_folder/Working/*RESULT.gips*”. This will allow GIPS to reuse related intermediate results in its “update” mode.

## 2.2 Parameter and result file

### 2.2.1 Project initialization

Usage: Java -jar -Xms3g GIPS.jar -init /path/to/project\_folder

GIPS will create the directory “/path/to/project\_folder”. This directory contains four sub-folders:

“*Scripts*”, for storing user scripts.

“*Working*”, for storing intermediate results and archiving GIPS result files.

“*Ref*”, for storing reference data, such as genome annotation files and library of phenotype-causing variants.

“*Data*”, for storing user-provided sequence alignment (SAM) files and variant call (VCF) files.

The “*project\_folder*” also contains two text files:

“*PROJECT.ini*”, which specifies the analysis procedure to be evaluated (detailed below);

“*Working/RESULT.gips*”, which presents GIPS results (detailed blow).

### 2.2.2 Analysis procedure specification (“*PROJECT.ini*”)

The “*PROJECT.ini*” file includes one “[*GLOBAL*]” section, followed by multiple “[*SAMPLE*]” sections. There is also a section named “[*SAMPLE\_LIST*]”, which lists all sample names. If the same parameter appears in both the “[*GLOBAL*]” section and a “[*SAMPLE*]” section, the “[*SAMPLE*]” specific specification overrides the “[*GLOBAL*]” specification, which applies only to that specific sample. “#” is used to add comments.

The “[*GLOBAL*]” section contains the following specifications.

*PROJECT*: Project name, which is automatically filled when a project is initiated.

*SNPEFF\_GENOME\_VERSION*: The genome version code used by SnpEff to annotate variants.  
This is a required parameter with no default value.

*REF\_GENOME\_ANNOTATION.GFF*: /path/to/genome\_annotation.gff. The genome annotation file that GIPS uses to select the “effective genomic region”. This is a required parameter with no default value. If only a file name is specified, GIPS looks for this file in “/path/to/project\_folder/Ref”.

*SNPEFF*: /path/to/program/SnpEff/folder. Note that this is the path to SnpEff program folder, not the path to the SnpEff executable (jar file). This is a required parameter with no default value. SnpEff can be downloaded at <http://snpeff.sourceforge.net/index.html>.

*VAR\_CALL\_SCRIPT*: /path/to/variant\_calling\_script. GIPS invokes user-supplied scripts to call variants from simulated sequencing reads. User-supplied variant calling scripts need to take two command line parameters. The first one (“\$1” in common Linux shell script) specifies a path to the input SAM file, and the second one (“\$2” in common Linux shell script) specifies a path to the output VCF file. This is a required parameter with no default value. If only a file name is specified, GIPS looks for this script in “/path/to/project\_folder/Script”.

*CANDIDATE\_CRITERION*: The minimal number of phenotype-exhibiting samples in which a candidate gene is expected to harbor variants. Defaults to the total number of samples (which reports only genes that harbor variants in all samples).

*EFF\_REGION*: Combination of the following regions, separated by “|”: promoter=length, CDS, 5UTR, 3UTR, SpliceSite=length. Example: “*EFF\_REGION=CDS|PROMOTER=1000*”. Defaults to “*CDS|SpliceSite=2|PROMOTER=1000*”. This parameter is case-insensitive.

*VAR\_FILTERS*: Variant filters to be used. “A”: Ancestry filter. “B”: Big difference filter. “E”: Effective region filter. “C”: Congestion filter. Example: “*VAR\_FILTERS*: EB”. Defaults to “*EBA*”. Note that the “control filter” will take effect if the “*CONTROL*” parameter is specified. The effective region filter (“E”) will always be enabled. See 1.2.2 for details.

*SCORE\_MATRIX*: The scoring matrix used for filtering non-synonymous SNPs. GIPS has 87 built-in scoring matrices, which are listed in “*/path/to/GIPS\_folder/config*”. Defaults to “DEFAULT”, which means that no scoring matrix is used and all non-synonymous variants are kept.

*MAX\_AA\_SCORE*: The maximum amino acid similarity score as in the user specified scoring matrix for a non-synonymous SNP to be kept by the big difference filter. Its default value is “0”.

*NUM\_SIM\_SNPS*: Number of artificial SNP to be simulated when estimating the variant calling sensitivity for a sample. Defaults to 5,000.

*CONTROL*: */path/to/control\_sample.vcf*. A single file containing all variants that are found in phenotype-absent control samples. If there are multiple control samples, please merge their observed variants into one VCF file.

After the “[*GLOBAL*]” section, in each “[*SAMPLE*]” section, the following parameters are specified.

*SAMPLE\_NAME*: Name of this sample. This is a required parameter with no default value.

*READS\_ALIGNMENT.SAM*: */path/to/sample.sam*. The reads alignment result of this sample. Note that the user-supplied variant calling script should be able to take this file as input and produce the variant calling result that is specified in the “*SAMPLE.VCF*” parameter. This parameter is required if “*SPECIFY\_HOMO\_VDS*”, “*SPECIFY\_HETERO\_VDS*”, and “*SPECIFY\_BVF*” are not used. If only a file name is specified, GIPS looks for this file in “*/path/to/project\_folder/Data/*”.

*SAMPLE.VCF*: */path/to/sample.vcf*. The variant calling result of this sample. Note that the user-supplied variant calling script should be able to produce this result file from the reads alignment file specified in the “*READS\_ALIGNMENT.SAM*” parameter. This parameter is required if “*SPECIFY\_HOMO\_VDS*”, “*SPECIFY\_HETERO\_VDS*”, and “*SPECIFY\_BVF*” are not used. If only a file name is specified, GIPS looks for this file in “*/path/to/project\_folder/Data/*”.

*SPECIFY\_HOMO\_VDS*: Do not estimate the variant detection sensitivity for homozygous variants from the real sequencing data. Use the specified value instead. This parameter is required if “*READS\_ALIGNMENT.SAM*” is not used.

*SPECIFY\_HETERO\_VDS*: Do not estimate the variant detection sensitivity for heterozygous variants from the real sequencing data. Use the specified value instead. This parameter is required if “*READS\_ALIGNMENT.SAM*” is not used.

*SPECIFY\_BVF*: Do not estimate the background variant frequency from the real sequencing data. Use the specified value instead. This parameter is required if “*READS\_ALIGNMENT.SAM*” is not used.

*VAR\_CALL\_SCRIPT*: Overrides the same parameter in the “[*GLOBAL*]” section, if specified.

*SCORE\_MATRIX*: Overrides the same parameter in the “[*GLOBAL*]” section, if specified.

*MAX\_AA\_SCORE*: Overrides the same parameter in the “[*GLOBAL*]” section, if specified.

*CONTROL*: Overrides the same parameter in the “[*GLOBAL*]” section, if specified.

*NUM\_SIM\_SNPS*: Overrides the same parameter in the “[*GLOBAL*]” section, if specified.

Please note that the three parameters, *SPECIFY\_HOMO\_VDS*, *SPECIFY\_HETERO\_VDS* and *SPECIFY\_BVF*, should always be used together. Otherwise, GIPS would report an error, as below.

```
Job start time: 2013-07-31 10:09:24
temporary folder is created
Project: Test
Directory: /nas2/wwt/GIPS/Test
-----
Gene Identification via Phenotype Sequencing (Version 1.1.5)
Copyright (c) 2013-2014, Zhongxu Zhu, Xin Chen. All Rights Reserved.
-----
SAMPLE_NAME:sample1
SPECIFY_HOMO_VDS:1
SPECIFY_BVF:0.03
SAMPLE.VCF:/nas2/wwt/GIPS/Test/Data/sample1/sample1.vcf
08:00:00.67 [sample1] SPECIFY_BVF, SPECIFY_HOMO_VDS and SPECIFY_HETERO_VDS parameters should be specified together
```

### 2.2.3 Result file (“*RESULT.gips*”)

“*RESULT.gips*” has five sections, which may not all appear. Whether a section will appear depends on the type of GIPS run.

The “[*Study Effectiveness*]” section shows the chance of reporting the true phenotype-associated gene with the current analysis protocol, and, the significance of violating Mendelian assumption, if no gene is reported or all candidate genes have failed validation.

The “[*Candidate Gene List*]” section shows all candidate genes. Detailed information about the variants in these candidate genes is given in the result file “*Working/CANDIDATE\_GENES.txt*”.



The “[*Sample Specific Sensitivity and Specificity*]” section shows, for each sample, the variant calling sensitivity (section 1.2.1), false ignorance rate (section 1.2.2), variant detection sensitivity (section 1.2), and background variant frequency (section 1.3).

The “[*Protocol*]” section records the analysis protocol specified in “*PROJECT.ini*”.

The “[*GIPS TRACEBACK*]” section encodes the meta-data of intermediate results produced in this GIPS run. These intermediate results can be used to speed up future GIPS runs in the “update” mode. Users should not modify information in this section.

## 2.2.4 Running GIPS

Usage: java -jar GIPS.jar [options]

Example: java -Xms3g -jar GIPS.jar -T <tool> -p /path/to/project\_folder

**Table 1.** GIPS software options

Options:		
-h (-H)		Show help.
-Test		Initiate a new project with the test setup. See the next chapter for the “Test” example.
-init	/path/to/project_folder	Initiate a new project.
-T	<gips vcs filter>	Select GIPS function. gips: full workflow; vcs: only estimate the variant calling sensitivity for each sample; filter: only use variant filters to remove likely unrelated variants. Defaults to “gips”.
-update		Run GIPS in “update” mode. GIPS will try to reuse intermediate results produced in previous runs.

## Chapter 3. Examples

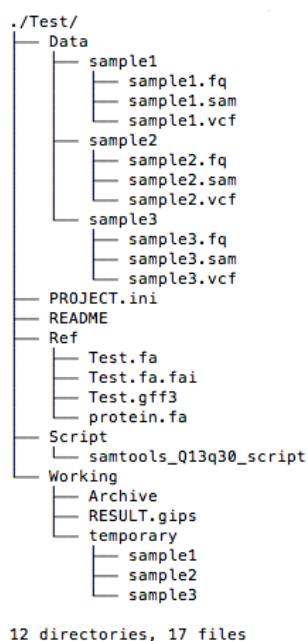
### 3.1 Test example

This example tests GIPS with synthesized artificial data.

Step 1: Initiate the test project.

Command: `java -jar GIPS.jar -Test`

The above command creates the GIPS project folder, “*Test*”. Its directory structure is as follows.



In “*Test/Data*”, there are three directories, “*sample1*”, “*sample2*” and “*sample3*”. These directories store the synthesized sequencing reads alignment results and variant calling results from three hypothetical samples. These artificial sequencing reads are generated by the ART simulator (Huang, Li et al. 2012).

In “*Test/Script*”, there is a variant calling script, which invokes SAMTools to call variants from mapped sequencing reads. Please modify this script accordingly to fit the user’s computing system. This script was used to call variants from the mapped reads of each hypothetical sample, and will be invoked by GIPS to estimate variant calling sensitivity (section 1.2.1). This script takes two command line parameters. The first one specifies a path to the input SAM file, and the second one specifies a

path to the output VCF file.

In “*project\_folder/Ref*”, there are two files. “*Test.fa*” provides the hypothetical reference genome sequence of the samples. “*Test.gff3*” annotates the hypothetical reference genome.

The “Working” directory has stored a sample GIPS result file, “*RESULT.gips*”, and some intermediate results. It is recommended that users also follow this convention to store intermediate results when developing their custom scripts to work with GIPS.

The parameter file “*PROJECT.ini*” specifies the analysis procedure to be executed and evaluated by GIPS (section 2.2.2).

Step 2: Setup SAMTools and SnpEff.

SAMTools can be downloaded at <http://samtools.sourceforge.net>.

Please make sure that “*samtools*”, “*bcftools*”, “*vcfutils.pl*” are all placed in the executable search path of the user system (\$PATH). Otherwise, please modify the “*Test/Script/samtools\_Q13q30\_script*” accordingly.

SnpEff can be downloaded at <http://snpeff.sourceforge.net/download.html>.

Unzip the downloaded package and set its folder path to the “*SNPEFF*” parameter in the “[*GLOBAL*]” section of the “*PROJECT.ini*” file and create a new folder named “Test” in “*SNPEFF/data*”.

Then you should put the “*project\_folder/Ref/Test.gff3*” file to the new Test folder and run the following command to create the *snpEffectPredictor.bin* file of Test.

Command: `java -jar /path/to/SNPEFF/snpeff.jar build -gff3 -v Test`

Step 3: Run GIPS to estimate variant calling sensitivity.

Command: `java -Xms3G -jar GIPS.jar -p Test -T vcs`

This command tests the GIPS function to estimate sample-specific variant calling sensitivities. The script “*Test/Script/samtools\_Q13q30\_script*” will be invoked to call variants from simulated reads alignment result, and GIPS will compute how much proportion of the simulated SNPs is recovered by this script (section 1.2.1).

The GIPS result file, “*Test/Working/RESULT.gips*”, will show contents like below.

```

=====
# Gene Identification via Phenotype Sequencing - Result File
#
# Job id: c8ad42c6ee5bb33346a2035eaf08b6aa
# Completion time: 2015-07-31 15:26:50
# Feature option '-T VCS'
=====

[Sample Specific Variant Calling Sensitivity (VCS)]
SAMPLE_NAME      HOMO_VCS      HETERO_VCS
sample1 0.661    0.263
sample2 0.638    0.351
sample3 0.647    0.383

#-----
# [Protocol]
#-----
# [GLOBAL]
# PROJECT          : Test
# REF_GENOME_ANNOTATION.GFF: /nas2/wwt/GIPS/Test/Ref/Test.off3
# SNPEFF_GENOME_VERSION   : Test

```

Step 4: Run GIPS to filter variants.

Command: `java -Xms3G -jar GIPS.jar -T filter -p Test`

This command tests the GIPS function to filter variants that are unlikely to associate with the target phenotype. The filtering procedure is specified in “*Test/PROJECT.ini*”. If successful, users will find three result files in “*Test/Working*”, with the names “sample1.filter.gips”, “sample2.filter.gips”, and “sample3.filter.gips”.

Step 5: Run a full GIPS workflow.

Command: `java -Xms3G -jar GIPS.jar -p Test -T gips`

This command tests the full GIPS workflow to call candidate genes from sequencing data. The hypothetical gene “*gene1\_gips*” is expected to appear in the “[*Candidate Gene List*]” section of the GIPS result file “*Test/Working/RESULT.gips*”. The GIPS result file will show contents like below.

```

=====
# Gene Identification via Phenotype Sequencing - Result File
#
# Job id: 138ef913f56f1a44e9b83ca8415abdda
# Completion time: 2015-07-31 15:31:46
# Feature option '-T gips'
=====

[Study Effectiveness]
Chance to report true phenotype-associated genes
If phenotype is recessive: 0.256
If phenotype is dominant: 0.0394

Significance of violating Mendelian phenotype assumption
If phenotype is recessive: 0.743
If phenotype is dominant: 1.0

Expected number of random genes that may be reported: 0.861

[Candidate Gene List]
#Details on the variants they harbor can be found in CANDIDATE_GENES.txt
GENE_ID GENE_NAME Chr EFF_REGION_LENGTH SIGNIFICANCE NUM_VARIANT NUM_SAMPLE
gene1 gene1_gips chrI 315 0.03706 8 3

[Sample Specific Sensitivity and Specificity]
Variant Calling Sensitivity (VCS)
SAMPLE_NAME HOMO_VCS HETERO_VCS
sample1 0.647 0.276
sample2 0.631 0.387
sample3 0.685 0.402

False Ignorance Rate (FIR)
SAMPLE_NAME FIR_FUNCTION FIR_STUDY FIR
sample1 0.0263 0.0 0.0263
sample2 0.0263 7.094E-4 0.027
sample3 0.0263 7.094E-4 0.027

Sample Variant Detection Sensitivity (VDS) (Measurement of sensitivity)
SAMPLE_NAME HOMO_VDS HETERO_VDS
sample1 0.629 0.268
sample2 0.613 0.376
sample3 0.666 0.391

```

In addition, a text file “*Test/Working/CANDIDATE\_GENES.txt*” will be created, which provides detailed information on the variants detected in “*gene1\_gips*” in each sample.

## 3.2 Identification of a *Pho2* suppressor gene in *Oryza Sativa*

### 3.2.1 Overview

Inorganic phosphorus (Pi) is an essential nutrient for plant growth and development, and Pi limitation is widely a constraint for crop yield in cultivated soils (Raghothama 1999). Understanding the molecular mechanisms of Pi uptake and utilization is important for breeding practices to improve nutrient efficiency in crops. The mutation of *PHOSPHATE2* (*PHO2*) was first described in *Arabidopsis*. The phenotype is over-accumulation of Pi in shoot tissues (Delhaize and Randall 1995). *Arabidopsis* *PHO2* was later characterized as an ubiquitin-conjugating E2 enzyme (Liu, Huang et al. 2012). *OsPHO2*, the *PHO2* homolog in rice (LOC\_Os05g48390), was also identified as an important regulator in phosphate translocation and homeostasis, which showed similar functions like *AtPHO2*. *Ospho2* mutant shows leaf tip necrosis and Pi accumulation largely in mature leaves (Wang, Ying et al. 2009, Hu, Zhu et al. 2011). In this study, we performed a forward genetic study to identify its potential suppressor genes in rice. A *pho2* Tos17 insertion mutant was obtained from the Rice Genome

Resource Center, Japan (<http://tos.nias.affrc.go.jp/>, Accession: NE8536). We derived a homozygous *pho2* mutant (HNE8536) from this line (Wang, Ying et al. 2009).

An EMS (ethyl methanesulfonate) induced mutant library was generated from the HNE8536 homozygous *pho2* mutant line. From the M2 population of ~15,000 lines grown in soil, three partial suppressor mutants showing an identical phenotype of Pi tolerance were obtained (M28, M29 and M249, Figure 5 (1A)). Because these mutants are produced by EMS treatment, they are considered unrelated and the ancestry filter (section 1.2.2.3) is enabled. The raw sequencing results can be retrieved from the Sequence Read Archive Database with accession SRP058881.

### 3.2.2 Run with empirical parameters

Step 1: Download raw sequencing data and convert to the FASTQ format.

**Table 2.** The Pho2 suppressor identification dataset.

SAMPLE NAME	SAMPLE ID	NCBI SRA database link
M28	SRS949736	<a href="http://www.ncbi.nlm.nih.gov/sra/?term=SRS949736">http://www.ncbi.nlm.nih.gov/sra/?term=SRS949736</a>
M29	SRS949738	<a href="http://www.ncbi.nlm.nih.gov/sra/?term=SRS949738">http://www.ncbi.nlm.nih.gov/sra/?term=SRS949738</a>
M249	SRS949741	<a href="http://www.ncbi.nlm.nih.gov/sra/?term=SRS949741">http://www.ncbi.nlm.nih.gov/sra/?term=SRS949741</a>

Sequencing reads data in SRA format need to be converted to FASTQ format using the SRA Toolkit (<http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=software>).

```
vdb-validate.2 /path/to/sample.sra
```

```
fastq-dump.2 --split-3 /path/to/sample.sra
```

Step2: Align sequencing reads to reference genome and call variants.

Paired-end sequencing reads are aligned to the *Oryza Sativa* Nipponbare reference genome version 7 (Kawahara, de la Bastide et al. 2013), using the Bowtie2 software (Langmead and Salzberg 2012). The reference genome FASTA file (all.chrs.con.fa) and annotation file (all.gff3) can be downloaded from

```
ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/
pseudomolecules/version_7.0/all.dir/
```



appropriately. In our system, these parameters are set to:

```
[GLOBAL]
PROJECT :GIPS-Rice
REF_GENOME_ANNOTATION.GFF : /path/to/pho2/reference/all.gff3
SNPEFF_GENOME_VERSION      : rice7
SNPEFF : /path/to/snpEff
```

Move the reads alignment results and variant calling results obtained in Step 2 into the GIPS project folder, “/path/to/GIPS-Rice/Data”. Provide the variant calling steps to GIPS as a script. See below for an example. This script takes two command line parameters. The first specifies the input sam file and the second specifies the output vcf file. Here, the below example script is named “*default-Q13-q0.sh*”. Move the variant calling script to “/path/to/rice/Script” and set its path in “*PROJECT.ini*”.

```
#!/bin/bash
# $1 /path/to/sam $2 /path/to/vcf. Intermediate files are recommended to store in /path/
to/project/Working/temporary. samtools version: 1.2

# sort and add read groups
java -Xms8g -jar /path/to/picard.jar AddOrReplaceReadGroups INPUT="$1" OUTPUT=/path/to/
GIPS-Rice/Working/temporary/btout.bam SORT_ORDER=coordinate RGPL=illumina RGLB="GIPS"
RGPU=temp RGSM="GIPS"

# remove duplicates
java -Xms8g -jar /path/to/picard.jar MarkDuplicates I=/path/to/GIPS-Rice/Working/
temporary/btout.bam O=/path/to/GIPS-Rice/Working/temporary/bt_markdup.bam M=/path/to/
GIPS-Rice/Working/temporary/duplication_metrics REMOVE_DUPLICATES=true AS=true

# samtools index
/path/to/samtools index /path/to/GIPS-Rice/Working/temporary/bt_markdup.bam

# samtools call variants
/path/to/samtools mpileup -ugf /path/to/all.chrs.con.fa /path/to/GIPS-Rice/Working/
temporary/bt_markdup.bam -go - | /path/to/bcftools call -vmO z -o - | /path/to/bcftools
filter -O v -o "$2"
```

After the above steps, the parameter file “*PROJECT.ini*” should look like below.



```

[GLOBAL]
PROJECT :GIPS-Rice
REF_GENOME_ANNOTATION.GFF : /path/to/pho2/reference/all.gff3
SNPEFF_GENOME_VERSION : rice7
SNPEFF : /path/to/snpEff
CANDIDATE_CRITERION : 3
VAR_CALL_SCRIPT : /path/to/GIPS-Rice/Script/default-Q13-q0.sh
EFF_REGION : CDS|SpliceSite=2|Promoter=1000
VAR_FILTERS : EAB
SCORE_MATRIX: DEFAULT
MAX_AA_SCORE:
NUM_SIM_SNPS : 10000

[SAMPLE_LIST]
M28
M29
M249

[SAMPLE]
SAMPLE_NAME : M28
SAMPLE.VCF : /path/to/GIPS-Rice/Data/M28/samtools_Q13_q0_noDPfilter.vcf
READS_ALIGNMENT.SAM : /path/to/GIPS-Rice/Data/M28/btout.sam

[SAMPLE]
SAMPLE_NAME : M29
SAMPLE.VCF : /path/to/GIPS-Rice/Data/M29/samtools_Q13_q0_noDPfilter.vcf
READS_ALIGNMENT.SAM : /path/to/GIPS-Rice/Data/M29/btout.sam

[SAMPLE]
SAMPLE_NAME : M249
SAMPLE.VCF : /path/to/GIPS-Rice/Data/M249/samtools_Q13_q0_noDPfilter.vcf
READS_ALIGNMENT.SAM : /path/to/GIPS-Rice/Data/M249/btout.sam

```

Step 4: Call candidate genes.

Command: `java -Xms3g -jar GIPS.jar -T gips -p GIPS-Rice`

This command will call candidate genes with the default analysis procedure. The default analysis procedure considers only coding sequences and splice sites (effective genomic region), and uses ancestry filter (section 1.2.2.3) and big difference filter (section 1.2.2.2). The default behavior of big difference filter is to remove only synonymous SNPs. Candidate genes are expected to harbor detected variants in all three samples.

As shown in “*/path/to/GIPS-Rice/Working/RESULT.gips*”, this default analysis procedure has ~91% chance to detect homozygous variant and ~81% chance to detect heterozygous variant in each sample. The combined likelihood of the true phenotype-associated gene harboring detected variants in all three samples is high (74%). These results are summarized in Table 3.

**Table 3.** Sample-wise study effectiveness measurements of the default analysis procedure. Homo\_VCS: homozygous variant calling sensitivity; Hetero\_VCS: heterozygous variant calling sensitivity; FIR: false ignorance rate; Homo\_VDS: homozygous variant detection sensitivity; Hetero\_VDS: Heterozygous variant detection sensitivity; BVF: background variant frequency.

Sample	Homo_VCS	Hetero_VCS	FIR	Homo_VDS	Hetero_VDS	BVF
M28	0.934	0.837	0.0167	0.918	0.822	1.51E-5
M29	0.883	0.812	0.0167	0.868	0.798	8.32E-6
M249	0.952	0.837	0.0167	0.936	0.822	8.46E-6

The default analysis procedure identified 28 candidate genes. Information on these genes and the mutations they harbor is detailed in the Appendix. In GIPS results, two files present these data. The candidate genes are listed in the “[Candidate Gene List]” section of the result file (*/path/to/GIPS-Rice/Working/RESULT.gips*), which looks like below. The variants that these candidate gene harbor are provided in a separate file (*path/to/GIPS-Rice/Working/CANDIDATE\_GENES.txt*).

```
[Candidate Gene List]
#Detailed information on the variants that these candidate genes harbor is provided in CANDIDATE_GENES.txt
GENE_ID GENE_NAME Chr EFF_REGION_LENGTH SIGNIFICANCE NUM_VARIANT NUM_SAMPLE
LOC_0s12g35520 LOC_0s12g35520 Chr12 1224 1.91128E-6 5 3
LOC_0s12g26100 LOC_0s12g26100 Chr12 1272 2.14343E-6 18 3
LOC_0s03g64310 LOC_0s03g64310 Chr3 1312 2.35057E-6 6 3
LOC_0s04g02550 LOC_0s04g02550 Chr4 1446 3.14017E-6 16 3
LOC_0s08g05950 LOC_0s08g05950 Chr8 1470 3.29787E-6 6 3
LOC_0s04g08690 LOC_0s04g08690 Chr4 1489 3.42638E-6 4 3
LOC_0s06g39744 LOC_0s06g39744 Chr6 1558 3.92082E-6 5 3
LOC_0s01g67710 LOC_0s01g67710 Chr1 1658 4.71779E-6 8 3
LOC_0s10g07040 LOC_0s10g07040 Chr10 2201 1.09421E-5 16 3
LOC_0s04g35000 LOC_0s04g35000 Chr4 2571 1.73379E-5 6 3
LOC_0s11g12710 LOC_0s11g12710 Chr11 2717 2.04153E-5 4 3
LOC_0s06g38270 LOC_0s06g38270 Chr6 3166 3.20722E-5 13 3
LOC_0s04g51840 LOC_0s04g51840 Chr4 3180 3.24924E-5 7 3
LOC_0s10g15210 LOC_0s10g15210 Chr10 3194 3.29161E-5 8 3
LOC_0s10g15170 LOC_0s10g15170 Chr10 3356 3.80851E-5 5 3
LOC_0s10g13550 LOC_0s10g13550 Chr10 3447 4.12086E-5 3 3
LOC_0s02g56510 LOC_0s02g56510 Chr2 3504 4.32479E-5 3 3
LOC_0s08g06280 LOC_0s08g06280 Chr8 3529 4.41627E-5 10 3
LOC_0s12g11570 LOC_0s12g11570 Chr12 3975 6.26682E-5 3 3
LOC_0s12g08110 LOC_0s12g08110 Chr12 4141 7.06662E-5 7 3
LOC_0s04g02520 LOC_0s04g02520 Chr4 4484 8.92358E-5 12 3
LOC_0s05g51770 LOC_0s05g51770 Chr5 4780 1.07595E-4 5 3
LOC_0s03g64320 LOC_0s03g64320 Chr3 4819 1.10183E-4 6 3
LOC_0s03g23760 LOC_0s03g23760 Chr3 4838 1.11458E-4 3 3
LOC_0s04g05104 LOC_0s04g05104 Chr4 5084 1.28838E-4 6 3
LOC_0s02g32690 LOC_0s02g32690 Chr2 5330 1.47884E-4 12 3
LOC_0s04g49890 LOC_0s04g49890 Chr4 5346 1.49183E-4 6 3
LOC_0s04g13510 LOC_0s04g13510 Chr4 5578 1.68841E-4 23 3
```

Rough running times for the above analysis procedures are listed in Table 4.

**Table 4.** Rough running times on a single CPU Intel Xeon E3-1230 server.

Procedure	Runtime
SRA Toolkit	~12min
Bowtie2 build index	~9min
Bowtie2 align	~2.5h
Picard sort and add groups	~45min
Picard rmdup	~40min
Samtools index	~2.5min
Samtools call variants	~3.5h
<b>Total</b>	<b>~ 8h</b>
<b>GIPS</b>	<b>1h</b>

### 3.2.3 Run with optimized parameters

The default analysis procedure identified 28 candidate genes, which are too many for experimental validation. Such a big number of identified candidates indicates the lack of specificity of the default analysis procedure. Therefore, next, we modify the default analysis procedure to improve its specificity.

Step 5: Modify the analysis procedure.

1) Use a more strict quality criterion in the variant calling step.

We increase the minimum base quality score from 13 to 15, and increase the minimum mapping quality score from 0 to 20. The updated variant calling procedure is as below.

```
/path/to/samtools mpileup -ugf /path/to/all.chrs.con.fa /path/to/pho2/dataset/
sample_markdup.bam -Q 15 -q 20 -go - | /path/to/bcftools call -vm0 z -o - | /path/to/
bcftools filter -O v -o /path/to/pho2/dataset/samtools_Q15_q20_noDPfilter.vcf
```

The updated procedure is then used to call variants from the actual sequencing reads. The resultant VCF files (i.e., “/path/to/pho2/Dataset/sample/samtools\_Q15\_q20\_noDPfilter.vcf”) should be moved to the “/path/to/GIPS-Rice/Data/sample/” folder. Meanwhile, create a new script to supply this updated procedure to GIPS. We name this script “samtools\_Q15\_q20.sh”, and put it in “/path/to/GIPS-Rice/Script”. This script is a modified version of “default-Q13-q0.sh” (see above), with the samtools parameters changed. This script takes two command line parameters. The first specifies the input sam file and the second specifies the output vcf file.

2) Use a more strict “big difference filter”.

In the “[GLOBAL]” section of “PROJECT.ini”, set:

SCORE\_MATRIX: PAM120

This change instructs the big difference filter to remove non-synonymous variants that result in similar amino acid changes with positive similarity scores in the PAM120 scoring matrix.

3) Shrink the effective genomic region to include only CDS regions and splice sites.

It is arguable that most phenotype-related de novo mutations happen in the CDS regions and splice sites. By considering only the CDS regions and splice sites, we reduce the number of detected variants that may confound the gene identification process.

In the “[GLOBAL]” section of “PROJECT.ini”, set:

EFF\_REGION: CDS|SpliceSite=2

The “*PROJECT.ini*” file is then updated to reflect these changes.

```
[GLOBAL]
PROJECT :GIPS-Rice
REF_GENOME_ANNOTATION.GFF : /path/to/pho2/reference/all.gff3
SNPEFF_GENOME_VERSION : rice7
SNPEFF : /path/to/snpEff
CANDIDATE_CRITERION : 3
VAR_CALL_SCRIPT : /path/to/GIPS-Rice/Script/samtools-Q15-q20.sh
EFF_REGION : CDS|SpliceSite=2
VAR_FILTERS : EAB
SCORE_MATRIX: PAM120
MAX_AA_SCORE:
NUM_SIM_SNPS : 10000

[SAMPLE_LIST]
M28
M29
M249

[SAMPLE]
SAMPLE_NAME : M28
SAMPLE.VCF : /path/to/GIPS-Rice/Data/M28/samtools_Q15_q20_noDPfilter.vcf
READS_ALIGNMENT.SAM : /path/to/GIPS-Rice/Data/M28/btout.sam

[SAMPLE]
SAMPLE_NAME : M29
SAMPLE.VCF : /path/to/GIPS-Rice/Data/M29/samtools_Q15_q20_noDPfilter.vcf
READS_ALIGNMENT.SAM : /path/to/GIPS-Rice/Data/M29/btout.sam

[SAMPLE]
SAMPLE_NAME : M249
SAMPLE.VCF : /path/to/GIPS-Rice/Data/M249/samtools_Q15_q20_noDPfilter.vcf
READS_ALIGNMENT.SAM : /path/to/GIPS-Rice/Data/M249/btout.sam
```

Step 6: Run GIPS in “update” mode to evaluate the modified analysis procedure.

Command: `java -Xms3g -jar GIPS.jar -T gips -p GIPS-Rice -update`

This command will call candidate genes with the modified analysis procedure.

As shown in “*/path/to/GIPS-Rice/Working/RESULT.gips*”, the modified analysis procedure has ~70% chance to detect homozygous variants and ~67% chance to detect heterozygous variants in each sample. The combined likelihood of the true phenotype-associated gene harboring detected variants in all three samples is 29%. These results are summarized in Table 5.

**Table 5.** Sample-wise study effectiveness measurements of the modified analysis procedure. Homo\_VCS: homozygous variant calling sensitivity; Hetero\_VCS: heterozygous variant calling sensitivity; FIR: false ignorance rate; Homo\_VDS: homozygous variant detection sensitivity; Hetero\_VDS: Heterozygous variant detection sensitivity; BVF: background variant frequency.

Sample	Homo_VCS	Hetero_VCS	FIR	Homo_VDS	Hetero_VDS	BVF
M28	0.916	0.879	0.196	0.735	0.705	5.89E-6
M29	0.819	0.773	0.196	0.657	0.62	3.63E-6

M249	0.897	0.836	0.196	0.72	0.671	2.86E-6
------	-------	-------	-------	------	-------	---------

In contrast to the default analysis procedure, the modified analysis procedure has a less chance to detect the true phenotype-associated variant in each sample. This results in a decrease of the combined likelihood that the true phenotype-related gene harbors detected variants in all three samples. On the other hand, the modified analysis procedure detects much less variants in each sample, only ~23% of those detected with the default analysis procedure. This decrease of confounding variants increases the confidence in the identification of phenotype-associated gene. As a result, the significance of the top candidate gene has increased from  $1.911 \times 10^{-6}$  to  $9.453 \times 10^{-7}$ , using the modified analysis procedure.

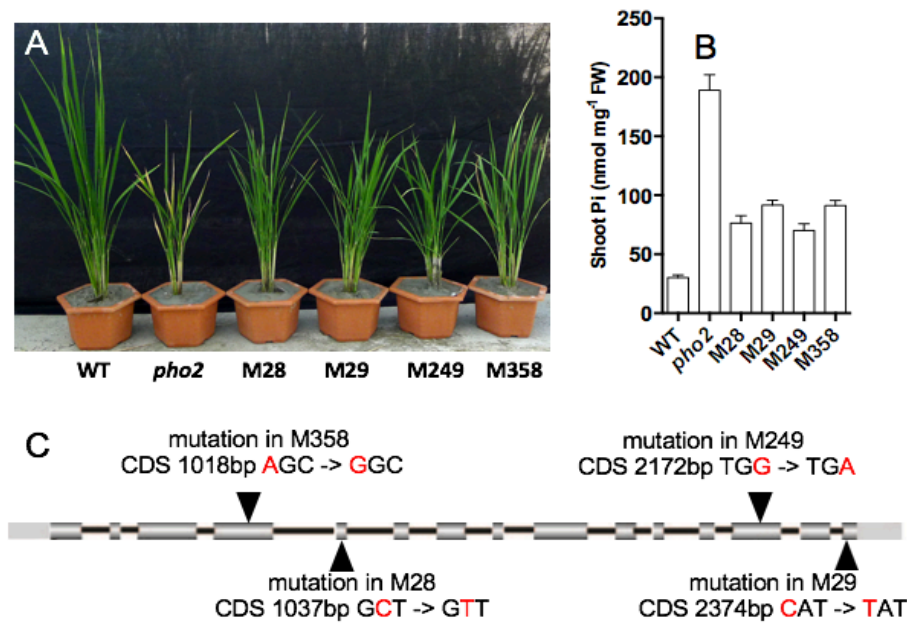
The modified analysis procedure reports only one candidate gene, *LOC\_Os02g56510* (OsPHO1;2). This candidate is a functional homology of the Arabidopsis AtPHO1 gene (Secco, Wang et al. 2012). AtPHO1 was proposed to function as a Pi transporter (Secco, Wang et al. 2012). A recent study has demonstrated that AtPHO1 is a crucial downstream component of AtPHO2. AtPHO2 modulates the degradation of AtPHO1 in endomembranes to maintain Pi homeostasis in Arabidopsis (Liang, Wang et al. 2014). Therefore, it is highly likely that OsPHO1;2 is the gene that suppressed the *pho2* mutant phenotype in rice. The variants detected in OsPHO1;2 are listed in Table 6.

**Table 6.** Variants detected in *OsPHO1;2*.

Sample	Pos	Ref	Alt	GT	Effect
M28	34611907	C	T	1/1	Missense (A:V)
M29	34614585	C	T	1/1	Missense (H:Y)
M249	34614218	G	A	1/1	Stop_gained

To further validate this gene-phenotype association, we screened another 5000 lines from the EMS-induced mutant library based on the HNE8536 homozygous *Ospho2* mutant line. One additional mutant (M358), which exhibited the same Pi tolerance phenotype, was obtained. As expected, we found a high-effect mutation Ser340Gly in *OsPHO1;2* in M358.

The mutant phenotypes and genotypes of M28, M29, M249, and M358 are shown in Figure 5. The phenotypes of the mutants were further validated by measuring their shoot Pi concentrations, and confirming their *Ospho2* Tos17 insertion (data not shown).



**Figure 5.** Plant phenotypes, Pi contents, and SNPs detected in the OsPHO1;2 region. (A) Phenotypes of 60-day-old plants grown in soil. WT: wild type Nipponbare; *pho2*: homozygous *pho2* mutant (HNE8536). (B) Shoot Pi contents of 40-day-old plants grown in +Pi (200  $\mu$ M) hydroponic medium (Error bars represent SE,  $n = 3$ ). (C) Variations detected in the OsPHO1;2 region.

### 3.2.4 Example discussion (strategy to optimize an analysis procedure)

This example provides case for discussing how an analysis procedure can be optimized based on the study effective measurements provided by GIPS, in particular, “the chance to report candidate genes that are truly associated with the phenotype” and “the significance of violating Mendelian assumption, when no candidate gene is reported or all candidates have failed validation”.

In the above example, the default analysis procedure reported 28 candidate genes (section 3.2.2), which are too many for validation. A more strict analysis procedure is therefore desired to identify candidates of higher confidence. For this reason, we optimized the analysis procedure toward accuracy and the optimized procedure identified only one candidate gene, which was subsequently confirmed with experiments. Although the optimized analysis procedure has a lower “chance to report the true phenotype-associated gene”, it is not informative as long as this procedure can identify a biologically sound candidate that is worthy of validation. The measurement “chance to report the true phenotype-associated gene” is intended to provide guidance on the next steps when an analysis procedure cannot identify any biologically sound candidate or all identified candidates have failed validation.

To identify a phenotype-associated gene, a more “strict” or “accurate” analysis procedure will only consider highly confident variations that are supported by a significant number of high quality

sequencing reads. Subsequent filtering process will also keep only variations that are very likely to produce the target phenotype. This “accurate” procedure will result in less number of possible variations that might produce the phenotype in each sample. Consequently, a candidate gene harboring variations in multiple samples will have a higher significance to associate with the phenotype. However, such an “accurate” procedure will also risk ignoring the true phenotype-causing mutations that are not supported by unequivocal evidence. This undesired ignorance can result in the true phenotype-associated gene harboring variations in an insufficient number of samples, which will fail the candidate gene criterion.

On the other hand, a more “permissive” or “comprehensive” analysis procedure will include more false positive variations and/or more less-likely phenotype-causing variations in each sample, which results in a higher chance that random genes may harbor variations in multiple samples. When the candidate gene criterion is met, the significance of a candidate to associate with the phenotype is also lower. Although a comprehensive analysis procedure is more unlikely to render the true phenotype-associated gene failing the candidate gene criterion, such a procedure is more likely to report phenotype-unrelated candidates, which demands significant extra effort in their validations.

Therefore, to most investigators, it is recommended to use a more accurate analysis procedure whenever possible. Accurate procedures produce highly confident candidates, which minimize the chance of failure in candidate validations. GIPS calculates a significance p-value for each candidate gene to associate with the phenotype. If there is at least one candidate gene unconfirmed and is not discouraged by other evidence (e.g., evidence that some of its variations might be false positive calls), it is advised to validate this candidate first. In this scenario, the study effectiveness measurement “chance to report the true phenotype-associated gene” is not informative. If it is low, it just indicates that this analysis procedure happens to fit the need of identifying this candidate gene very well. On the other hand, if an analysis procedure produces many candidates, this procedure is probably not accurate enough. Investigators are advised to try more aggressive approaches to further increase the confidence of the reported candidates.

Though it is always more advisable to use a accurate analysis procedure as long as it can identify biologically sound candidate genes for validation, in cases that the protocol is too strict to identify any candidate or all identified candidates have failed validation, the two study effectiveness measurements, “chance to report the true phenotype-associated gene” and “significance of violating Mendelian assumption”, may provide a guidance on the next steps. If the chance to report phenotype-associated gene is low, the analysis procedure is likely too strict and needs relaxation. Investigators may consider validating more candidates reported by a more permissive analysis procedure, or sequencing more phenotype-exhibiting samples to increase the support of the phenotype-associated gene.



Conversely, if an investigator has sequenced a large number of phenotype-exhibiting samples and/or have validated many candidates, and the phenotype-associated gene is still at large, the study effectiveness measurement, “significance of violating Mendelian assumption”, may advise on the next steps. If this significance is low ( $p > 0.05$ ), there is no compelling evidence that the phenotype is controlled by multiple genes and the investigator is still advised to add samples, validate more candidates, or to relax the analysis procedure to identify more candidates. If this significance is high ( $p < 0.05$ ), the investigator is advised to re-examine the phenotype-exhibiting samples included in the study.

For a qualitative trait, though it is possible that multiple genes can produce similar phenotypes, it is unlikely that two genes produce exactly the same phenotype. This is because most, if not all, genes are pleiotropic. Disruptions of different genes might produce the same major effect, but each will also produce dissimilar “side effects”. Therefore, arguably, key to the success of a sequencing-based direct gene cloning study is the definition of a proper set of phenotype criteria, which can identify mutants of the same gene. The stricter the phenotype criteria are, the more likely that the included samples are mutants of the same gene. When GIPS reports a high significance of violating Mendelian assumption, investigators are advised to re-confirm the phenotypes of the samples included in the study. If there is no doubt, investigators are advised to consider using a more strict set of phenotype criteria for this study, which examine more minor phenotypic traits and can distinguish mutants of different but functionally related genes.

In general, the approach of sequencing based direct cloning in forward genetics study is expected to gain popularity. The reasons are two folds. First, this approach does not require the generation of cross or backcross populations, which significantly accelerates the gene identification process. Although this time advantage may come with more effort spent in screening mutant library to obtain multiple unrelated mutants of the same phenotype, library screenings in typical forward genetics studies require only bare-eye observation. The cost of screening a larger library is usually acceptable. Furthermore, rapid development of automated phenotyping technologies makes it possible to screen large libraries for minor phenotypes that cannot be easily observed (Pardo-Martin, Allalou et al. 2013). Second, because this approach does not require the creation of a population, it is free from the related limitations. This approach is readily applicable in identification of genes that are important in organ development (the disruption of which breaks the plant life cycle), and reproductive development (the disruption of which impedes crossing operation).

In this context, GIPS provides guidance on the effective design and execution of a sequencing based direct cloning study. It is different from other gene prioritization software, such as ANNOVAR (Wang, Li et al. 2010), which scores genes and variants to provide a rank. These priority scores do not

advise, when no phenotype-associate gene can be identified, whether an investigator should change the analysis procedure, validate more candidate genes, add more samples, or re-examine the phenotype criteria used in the study. GIPS implements a probabilistic framework that models the entire process of sequencing based direct cloning study. Within this framework, other gene prioritization software focusing on removing genes/variants that are unlikely to associate with the phenotype can be integrated with the GIPS workflow as additional gene/variant filters.

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## Abbreviations

Term	Abbreviation/Illustration	Section
BVF :	Background variant frequency	1.3
CDS:	Coding sequence	
ClinVar:	Clinical variant database	1.2.2
FIR:	False ignorance rate	1.2.2
GIPS:	Gene identification via phenotype sequencing	
SAM :	Sequence Alignment/Map Format	
VDS:	Variant detection sensitivity	1.2
VCS:	Variant calling sensitivity	1.2.1
VCF:	Variant Call Format	
<i>PROJECT.ini</i>	Parameter file to specify the analysis procedure that will be executed and evaluated by GIPS	2.2.2
<i>RESULT.gips</i>	GIPS result file	2.2.3
<i>CANDIDATE_G ENES.txt</i>	Detailed information about the variants found in candidate genes	2.2.3
Effective genomic region	Genomic regions that are considered by GIPS in gene identification	1.2.2.1
Effective region filter	Discards variants that are outside the user-specified “effective genomic region”	1.2.2.1
Ancestry filter	Discards variants that are detected in more than one phenotype-exhibiting samples (when all samples are known unrelated)	1.2.2.3
Big difference filter	Discards non-synonymous variants that result in similar amino acids changes	1.2.2.2
Congestion filter	Discards a variant if there is another variant located in its vicinity (11bp region centering on it)	1.2.2.4
Control filter	Discards variants observed in phenotype-absent control samples	1.2.2.5

Appendix: The 28 candidate genes and their mutations discovered using a typical analysis procedure

[illegible]

	mutation NO 9	29	Chr10	3696070	T	A	44	0/1	
	mutation NO 10	29	Chr10	3696106	C	A	46	0/1	
	mutation NO 11	29	Chr10	3696905	A	G	44	0/1	missense_variant(M:V)
	mutation NO 12	29	Chr10	3696909	T	G	45	0/1	missense_variant(F:C)
	mutation NO 13	29	Chr10	3696976	A	T	65	0/1	missense_variant(E:D)
	mutation NO 14	29	Chr10	3698157	A	G	51	0/1	missense_variant(I:V)
	mutation NO 15	29	Chr10	3698439	A	G	64	0/1	missense_variant(N:D)
	mutation NO 16	29	Chr10	3698454	G	A	67	0/1	missense_variant(V:I)
10	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os04g35000	LOC_Os04g35000	Chr4	2571	1.73E-05	6	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr4	21270863	TTTGGGCCCAAAA/	T	4	1/1	
	mutation NO 2	249	Chr4	21270877	A	ACATTG	4	1/1	
	mutation NO 3	28	Chr4	21270864	TTGGGCCCAAAA/	T	20	1/1	
	mutation NO 4	29	Chr4	21270860	CA	CATTGAA	27	1/1	
11	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os11g12710	LOC_Os11g12710	Chr11	2717	2.04E-05	4	3	retrotransposon, putative, centromere-specific	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr11	7182119	.	CTTTTTTTTT	39	0/1	
	mutation NO 2	28	Chr11	7181320	.	G	A	215	0/1
	mutation NO 3	29	Chr11	7181202	.	C	G	121	0/1
	mutation NO 4	29	Chr11	7181756	.	T	TTTCGA	25	0/1
12	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os06g38270	LOC_Os06g38270	Chr6	3166	3.21E-05	13	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr6	22640315	G	A	61	0/1	missense_variant(P:S)
	mutation NO 2	249	Chr6	22640396	T	A	54	0/1	stop_gained
	mutation NO 3	28	Chr6	22639587	G	A	86	0/1	missense_variant(A:V)
	mutation NO 4	28	Chr6	22640068	G	A	84	0/1	stop_gained
	mutation NO 5	28	Chr6	22640070	G	A	85	0/1	missense_variant(P:L)
	mutation NO 6	28	Chr6	22642280	G	A	85	0/1	
	mutation NO 7	28	Chr6	22642418	G	A	99	0/1	
	mutation NO 8	28	Chr6	22642419	C	T	97	0/1	
	mutation NO 9	28	Chr6	22642449	C	T	91	0/1	
	mutation NO 10	28	Chr6	22642466	G	A	89	0/1	
	mutation NO 11	28	Chr6	22642469	T	C	92	0/1	
13	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os04g51840	LOC_Os04g51840	Chr4	3180	3.25E-05	7	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr4	30743500	ACC	AC	12	1/1	
	mutation NO 2	28	Chr4	30743409	A	G	13	1/1	
	mutation NO 3	29	Chr4	30743033	T	G	5	1/1	
	mutation NO 4	29	Chr4	30743042	C	A	4	1/1	
	mutation NO 5	29	Chr4	30743059	A	AC	4	1/1	
14	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os10g15210	LOC_Os10g15210	Chr10	3194	3.29E-05	8	3	retrotransposon protein, putative, unclassified	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr10	8054116	A	G	26	0/1	missense_variant(M:T)
	mutation NO 2	249	Chr10	8054122	C	G	26	0/1	missense_variant(R:T)
	mutation NO 3	249	Chr10	8054928	G	A	38	0/1	
	mutation NO 4	28	Chr10	8054332	A	G	12	0/1	missense_variant(L:P)
	mutation NO 5	28	Chr10	8054522	A	T	24	0/1	
15	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os10g15170	LOC_Os10g15170	Chr10	3356	3.81E-05	5	3	retrotransposon protein, putative, Ty3-gypsy subclass	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr10	8068176	T	C	64	0/1	missense_variant(T:A)
	mutation NO 2	28	Chr10	8066893	T	C	39	0/1	missense_variant(Q:R)
	mutation NO 3	28	Chr10	8068428	C	T	131	0/1	splice_acceptor_variant&intron_variant
	mutation NO 4	28	Chr10	8068721	G	A	93	0/1	missense_variant(P:L)
	mutation NO 5	29	Chr10	8067941	T	G	43	0/1	missense_variant(N:T)
16	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os10g13550	LOC_Os10g13550	Chr10	3447	4.12E-05	3	3	putative, expressed	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr10	7337673	TT	TTGGCAAAAT	5	1/1	
17	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os02g56510	LOC_Os02g56510	Chr2	3504	4.32E-05	3	3	Full=Protein PHO1-2; Short=OsPHO1;2	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr2	34614218	G	A	41	1/1	stop_gained
18	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os08g06280	LOC_Os08g06280	Chr8	3529	4.42E-05	10	3	SIMULATING DISEASE 1; Short=OsLSD1; AltName:	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr8	3463799	AAAAGGA	AA	51	0/1	
19	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g11570	LOC_Os12g11570	Chr12	3975	6.27E-05	3	3	[Oryza sativa Japonica Group]	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	6272224	T	A	4	1/1	
20	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4141	7.07E-05	7	3	[Oryza sativa Japonica Group]	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
21	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
22	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
23	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
24	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
25	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
26	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
27	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
28	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
29	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
30	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
31	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
32	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
33	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
34	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
35	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
36	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
37	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
38	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	
39	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os12g08110	LOC_Os12g08110	Chr12	4132130	CGAAC	3AACAAACGTGA/	40	0/1	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr12	4132282	C	T	51	0/1	</

20	mutation NO 3	28	Chr12	4132144	T	G	44	0/1	
	mutation NO 4	29	Chr12	4131953	C	T	6	0/1	
	mutation NO 5	29	Chr12	4131969	A	G	8	0/1	
	mutation NO 6	29	Chr12	4131980	C	T	9	0/1	
	mutation NO 7	29	Chr12	4134637	C	T	36	0/1	missense_variant(P:L)
21	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os04g02520	LOC_Os04g02520	Chr4	4484	8.92E-05	12	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr4	925275	T	C	45	0/1	missense_variant(D:G)
	mutation NO 2	249	Chr4	925293	C	T	39	0/1	missense_variant(S:N)
	mutation NO 3	28	Chr4	925767	A	T	94	0/1	missense_variant(L:Q)
	mutation NO 4	28	Chr4	925770	C	T	99	0/1	missense_variant(S:N)
	mutation NO 5	28	Chr4	925777	C	T	103	0/1	missense_variant(D:N)
	mutation NO 6	29	Chr4	925102	T	C	37	0/1	missense_variant(I:V)
	mutation NO 7	29	Chr4	925137	A	G	41	0/1	missense_variant(V:A)
	mutation NO 8	29	Chr4	925441	T	C	40	0/1	missense_variant(S:G)
	mutation NO 9	29	Chr4	925451	T	G	43	0/1	missense_variant(E:D)
	mutation NO 10	29	Chr4	926266	T	C	37	0/1	missense_variant(M:V)
	mutation NO 11	29	Chr4	926277	G	C	40	0/1	missense_variant(S:W)
	mutation NO 12	29	Chr4	926290	C	T	35	0/1	missense_variant(E:K)
22	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os05g51770	LOC_Os05g51770	Chr5	4780	1.08E-04	5	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr5	29709125	A	G	56	0/1	
	mutation NO 2	28	Chr5	29708324	G	A	46	0/1	
	mutation NO 3	29	Chr5	29704719	G	A	56	0/1	missense_variant(P:L)
23	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os03g64320	LOC_Os03g64320	Chr3	4819	1.10E-04	6	3	expressed protein	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr3	36347342	GTAT	GTATCTAT	34	0/1	
	mutation NO 2	249	Chr3	36347351	TATG	TATGGATG	33	0/1	
	mutation NO 3	28	Chr3	36347346	GTAT	GTATTTAT	41	0/1	
	mutation NO 4	28	Chr3	36347359	TATG	TATGGATG	38	0/1	
24	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os03g23760	LOC_Os03g23760	Chr3	4838	1.11E-04	3	3	retrotransposon protein, putative, Ty3 gypsy subclass	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr3	13462020	A	G	71	0/1	missense_variant(H:R)
	mutation NO 2	28	Chr3	13463366	G	A	124	0/1	missense_variant(R:H)
25	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os04g05104	LOC_Os04g05104	Chr4	5084	1.29E-04	6	3		
	SAMPLE	CHROM	POS	ALT	SEQ-DEPTH	GT	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr4	2526021	TG	T	4	1/1	frameshift_variant
	mutation NO 2	28	Chr4	2525974	G	T	14	1/1	missense_variant(L:M)
	mutation NO 3	28	Chr4	2530633	GT	G	30	1/1	frameshift_variant
	mutation NO 4	29	Chr4	2525859	G	T	6	1/1	missense_variant(A:E)
26	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os02g32690	LOC_Os02g32690	Chr2	5330	1.48E-04	12	3	Short=OsABCG41; AltName: Full=Pleiotropic drug resistance	
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr2	19409046	G	T	46	0/1	missense_variant(A:E)
	mutation NO 2	249	Chr2	19409047	C	T	47	0/1	missense_variant(A:T)
	mutation NO 3	249	Chr2	19409049	C	T	47	0/1	missense_variant(R:H)
	mutation NO 4	28	Chr2	19420231	TGCGGC	TGC	72	0/1	inframe_deletion
	mutation NO 5	28	Chr2	19420463	G	C	82	0/1	missense_variant(Q:E)
	mutation NO 6	28	Chr2	19420634	TGCG	TG	80	0/1	inframe_deletion
	mutation NO 7	28	Chr2	19420673	G	A	74	0/1	missense_variant(P:S)
	mutation NO 8	28	Chr2	19421642	A	C	24	0/1	
	mutation NO 9	28	Chr2	19421658	G	A	30	0/1	
	mutation NO 10	28	Chr2	19421693	C	G	40	0/1	
27	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os04g49890	LOC_Os04g49890	Chr4	5346	1.49E-04	6	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr4	29743379	T	TAAAA	33	1/1	
	mutation NO 2	249	Chr4	29743385	G	GA	33	1/1	
	mutation NO 3	249	Chr4	29743386	T	TA	35	1/1	
28	GENE_ID	GENE_NAME	Chr	EFF_REGION_LENGTH	SIGNIFICANCE	NUM_VARIANT	NUM_SAMPLE	DESCRIPTION	
	LOC_Os04g13510	LOC_Os04g13510	Chr4	5578	1.69E-04	23	3		
	SAMPLE	CHROM	POS	REF	ALT	SEQ-DEPTH	ZYGOSITY	PREDICTED EFFECTS	
	mutation NO 1	249	Chr4	7534243	T	C	68	0/1	missense_variant(I:V)
	mutation NO 2	249	Chr4	7534259	C	G	68	0/1	missense_variant(L:F)
	mutation NO 3	249	Chr4	7534864	T	C	65	0/1	missense_variant(N:D)
	mutation NO 4	249	Chr4	7537722	A	G	57	0/1	
	mutation NO 5	249	Chr4	7537724	G	A	57	0/1	
	mutation NO 6	249	Chr4	7537750	T	C	57	0/1	
	mutation NO 7	249	Chr4	7537906	C	T	51	0/1	
	mutation NO 8	249	Chr4	7537956	G	T	61	0/1	
	mutation NO 9	28	Chr4	7533456	A	C	67	0/1	missense_variant&splice_region_variant(F:V)
	mutation NO 10	28	Chr4	7534789	T	C	89	0/1	missense_variant(K:E)
	mutation NO 11	28	Chr4	7537271	T	A	107	0/1	missense_variant(Q:L)
	mutation NO 12	28	Chr4	7537305	C	T	99	0/1	missense_variant(E:K)
	mutation NO 13	28	Chr4	7537667	C	T	71	0/1	
	mutation NO 14	29	Chr4	7533273	T	C	41	0/1	missense_variant(N:D)
	mutation NO 15	29	Chr4	7535269	T	G	36	0/1	missense_variant(K:Q)
	mutation NO 16	29	Chr4	7537422	G	C	55	0/1	missense_variant(P:A)
	mutation NO 17	29	Chr4	7537435	T	G	52	0/1	missense_variant(E:D)
	mutation NO 18	29	Chr4	7537561	C	T	64	0/1	
	mutation NO 19	29	Chr4	7537571	G	A	62	0/1	
	mutation NO 20	29	Chr4	7537577	A	G	59	0/1	
	mutation NO 21	29	Chr4	7537598	C	T	66	0/1	
	mutation NO 22	29	Chr4	7538425	T	A	47	0/1	
	mutation NO 23	29	Chr4	7538426	A	C	47	0/1	