

LTR_retriever User Manual

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Questions and Issues Please See: https://github.com/oushujun/LTR_retriever

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1 Introduction

LTR_retriever is a command line program (in Perl) for accurate identification of LTR retrotransposons (LTR-RTs) from outputs of *LTRharvest* (1), *LTR_FINDER* (2), and *MGEscan-LTR* (3, 4) and generation of a non-redundant LTR-RT library for genome annotations.

As one of the most prevalent transposable elements (TEs), LTR-RT comprises the largest portion of most plant genomes (5). Due to the sequence diversity of LTR-RTs, identification of such elements based on sequence homology is inefficient. Instead, LTR-RTs are conserved in terms of element structure across different species. Several programs have been developed to search for LTR-RTs using relevant structural characteristics. These programs are very sensitive; however, they are not very accurate and specific for LTR-RT identifications. *LTR_retriever* was developed to address the accuracy and specificity needs, with several new functions to facilitate genome annotation and other downstream studies.

LTR_retriever aims to identify high-quality LTR-RT exemplars (**Figure 1A**) that are intact and non-redundant from a variety of LTR-RT candidates. To retain sensitivity, sequences of nested LTRs and truncated LTRs (**Figure 1CD**) that are not represented by intact LTR-RTs will also be included in the exemplar. This package excludes the vast majority of the non-LTR false positives. The most common false positives were introduced by two adjacent non-LTR repeats which are found as

SINEs, LINEs, DNA TEs, or solo-LTRs that are derived from different elements (**Figure 3**). In addition, *LTR_retriever* excludes non-LTR open reading frames derived from LINEs, DNA TEs, or plant coding sequences to reduce misannotations of non-LTR coding sequences as LTR elements. *LTR_retriever* identifies and removes LTR-RT nested insertions in the identified intact LTR-RTs, which also reduces library redundancy. This program can also accurately identify rare non-canonical LTR-RTs that have terminal motifs different from the canonical 5'-TG..CA-3' motif. The program was built with a variety of Perl scripts that can be utilized for downstream analyses.

1.1 Main features of LTR_retriever

- A command line Perl program;
- Supports multi-threading;
- Identifies intact LTR-RTs with accurate boundaries;
- Identifies rare LTR-RTs with non-canonical (non-TGCA') motifs;
- Supports multiple inputs: *LTRharvest*, *LTR_FINDER*, and/or *MGEScan_LTR*;
- Sequence input: **FASTA** format (contigs, scaffolds, genomes, corrected PacBio reads, and etc.);
- Output: a non-redundant LTR-RT library (**FASTA**), **GFF3** for all intact LTR-RTs, whole-genome LTR-RT annotation (**GFF**), and a comprehensive table.

2 The Structure and Characteristics of LTR-RTs

The structure of an LTR retrotransposon (LTR-RT) is characterized by long terminal repeat ranging from 75 bp to 5000 bp (**Figure 1A**). The region between the 5' LTR and 3' LTR is termed the internal region, which encodes proteins for transposition. At the very termini of the LTRs are the bi-nucleic motifs, which is 5'-TG..CA-3' in most cases. However, various other motifs have been detected in the sacred lotus (*Nelumbo nucifera*) genome and in the rice (*Oryza sativa*) genome during our manual annotation, and also found in other studies (e.g., *Tos17* (6) ; *AtRE1* (7); and *TARE1* (8)). Flanking the terminal motifs is the target site duplication (TSD), which is generated by staggered cuts from integrase activity (**Figure 2**) during LTR-RT insertion. TSDs are typically 5 bp in plants but could vary between 3-6 bp, and the 5' and 3' TSD should be identical because of the mechanism of their formation (**Figure 2**). The recently inserted LTR-RT has a highly similar LTR region that is recognizable by sequence alignment, which is the primary searching scheme for LTR search programs (1, 2, 4, 9). However, if two highly similar repetitive elements other than LTR (e.g., DNA, LINE, SINE, solo-LTR, tandem repeat, etc.) are located close to each other (**Figure 3**), searching tools may falsely choose them and report them as LTR-RT candidates. These are the most frequent false positives that occur in *de novo* searches for LTR-RTs. Given that LTR-RTs are following the "copy-and-paste" duplication scheme, the regions flanking the newly inserted LTR-RT are unlikely to be identical to the termini of the internal region. For example, in an intact LTR-RT (**Figure 1A**), region "a" is not identical to region "c", and region "b" is not identical to region "d". Thus, by aligning the flanking regions of the two LTR fragments (**Figure 3**), *LTR_retriever* can obtain the boundary information for the candidate.

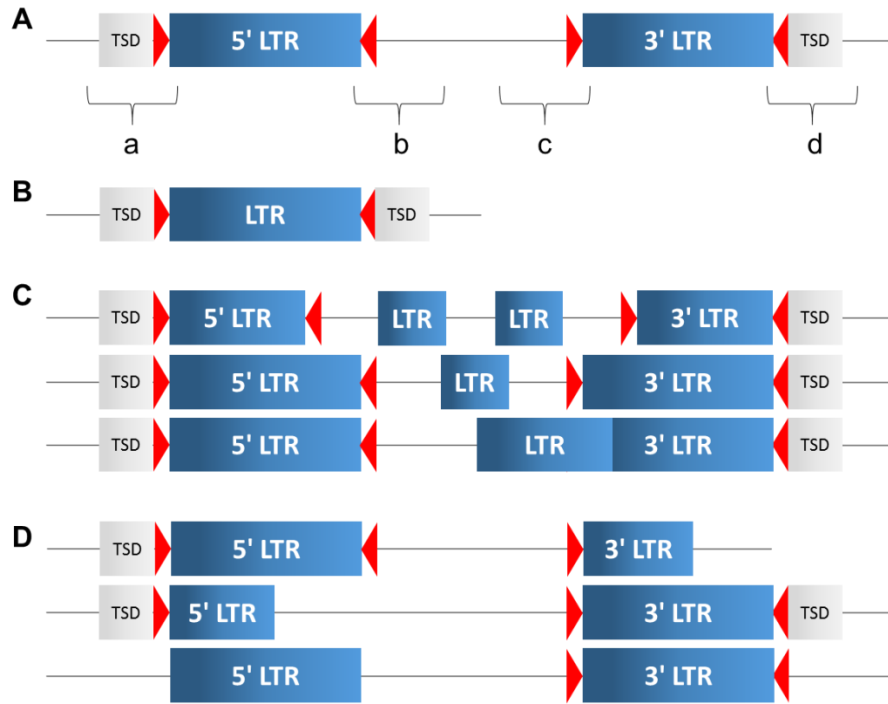


Figure 1. The structures of LTR retrotransposons (LTR-RT). Gray boxes: target site duplications (TSD); red triangles: LTR motifs; blue boxes: long terminal repeat (LTR); sequence between 5' LTR and 3' LTR denotes the internal region. **(A)** The structure of an intact LTR-RT. Regions a, b, c, and d are main targets analyzed by *LTR_retriever*. **(B)** The structure of a solo-LTR. **(C)** The structures of nest-inserted LTR-RTs. **(D)** The structures of truncated LTR-RTs. Drawing is not on scale.

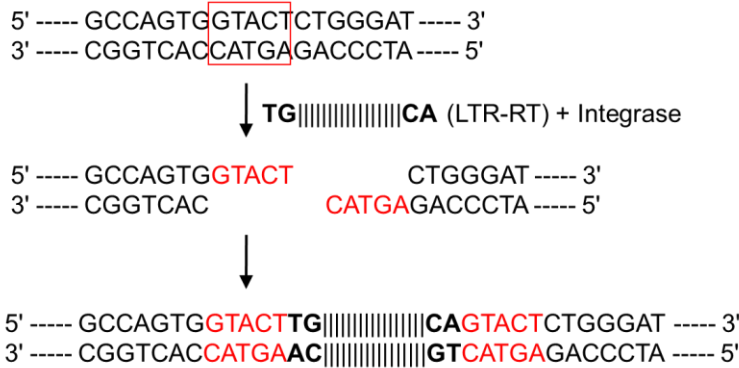


Figure 2. Formation of target site duplications (TSD). Integrases coded by LTR-RTs generate staggered cuts (in this case 5'-GTACT-3') on the sequence before new LTR-RT insertions. By gap filling and sequence ligation, a pair of TSD is formed flanking the newly inserted LTR.

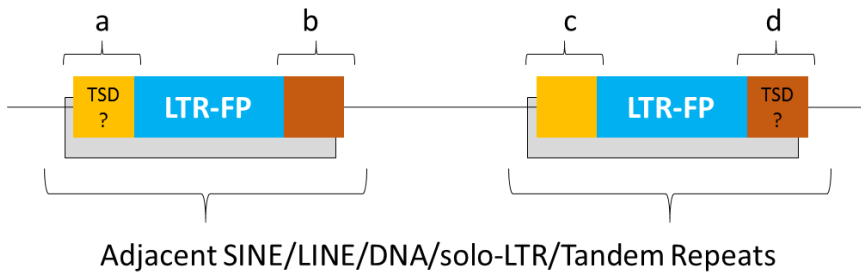


Figure 3. The most common false positives in *de novo* searches for LTR-RTs. Gray boxes: two closely positioned SINE/LINE/DNA elements/solo-LTRs/tandem repeats. Light blue boxes: false LTR regions reported by *de novo* searches. The false positive also has TSD-like structure but commonly has extended sequence identity on one or both termini (orange and brown boxes).

3 Workflow of *LTR_retriever*

In *LTR_retriever*, there are eight modules developed to screen and filter out false positives and construct non-redundant LTR exemplars (**Figure 4**). More details can be found in our article.

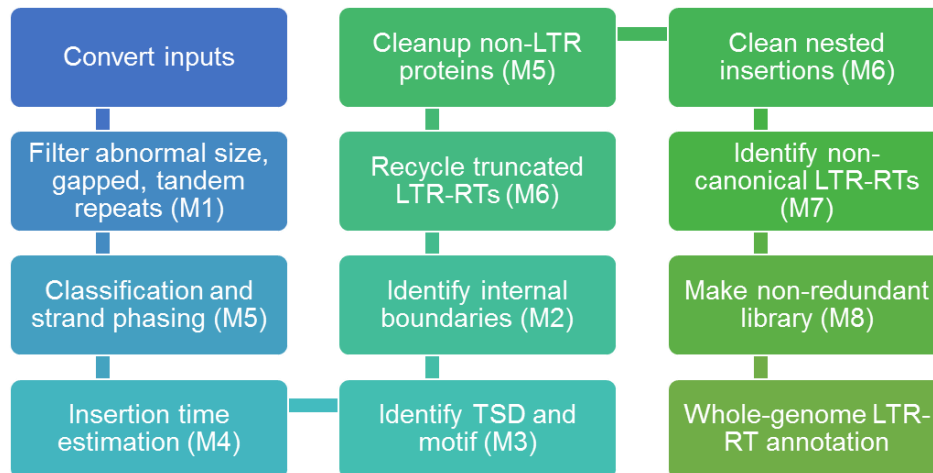


Figure 4. Workflow of *LTR_retriever*. Modules 1-8 are indicated in parentheses.

4 Installation

LTR_retriever is a command line Perl program that incorporates several programs for analysis and runs in UNIX-like systems. These programs include:

- BLAST+ (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/>),
- CDHIT (<http://weizhongli-lab.org/cd-hit/>) OR BLAST (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/legacy/2.2.25/>),
- HMMER (<http://hmmer.org/>), and
- RepeatMasker (<http://www.repeatmasker.org/>).

To run *LTR_retriever*, you need to provide the paths to the following dependent programs:

- *makeblastdb*, *blastn*, and *blastx* in the BLAST+ package,
- *cd-hit-est* in the CDHIT package OR *blastclust* in the BLAST package,
- *hmmsearch* in the HMMER package, and
- *RepeatMasker*

If the above programs are all accessible through ENV in the UNIX-like system (i.e. paths exported to **.bashrc**), no installation is needed. Otherwise, users need to modify the **paths** file under the **your_path_to/LTR_retriever/** directory. If specifying a path, the required program(s) must be directly contained in that path but not in any subdirectories.

For example:

Edit the **paths** file using *vi/vim* (you may use other text editors such as *emacs*)

```
vi /your_path_to/LTR_retriever/paths
```

Modify the following lines

```
BLAST+=/your_path_to/BLAST+2.2.30/bin/
RepeatMasker=/your_path_to/RepeatMasker4.0.0/
HMMER=/your_path_to/HMMER3.1b2/bin/
CDHIT=/your_path_to/CDHIT4.6.1/
BLAST=/your_path_to/BLAST2.2.26/bin/ #not required if CDHIT provided
```

Save changes to **paths** and exit. The installation is done.

5 Inputs

Two types of inputs are needed for *LTR_retriever*:

1. Genomic sequence
2. LTR-RT candidates

5.1 Genomic sequence

The sequence must be in **FASTA** format. Users should backup the original input **FASTA** file because *LTR_retriever* will modify sequence names that are longer than 20 characters to fit the naming space of *RepeatMasker*. Thus, **FASTA sequence names are recommended to be less than 20 characters without spaces and special punctuation marks other than dots (.) and underscores (_)**. For long sequencing reads (e.g., PacBio), self-corrected reads are needed unless the sequence error rate is lower than 10%.

5.2 LTR-RT candidates

LTR_retriever takes multiple LTR-RT candidate inputs including the standard output of *LTRharvest*, the standard output of *LTR_FINDER*, and the candidate output of *MGEScan-LTR*. Users need to obtain the input file(s) from the aforementioned programs before running *LTR_retriever*. Either a single input source or a combination of multiple inputs is acceptable. The following command lines provide examples and suggestions to obtain inputs.

Input from *LTRharvest* (a program of GenomeTools (10)): (“\” indicates this line and next

line belong to the same command line. Please delete “\” if you run into errors.)

```
gt suffixerator \  
  -db genome.fa \  
  -indexname genome.fa \  
  -tis -suf -lcp -des -ssp -sds -dna  
gt ltrharvest \  
  -index genome.fa \  
  -similar 90 -vic 10 -seed 20 -seqids yes \  
  -minlenltr 100 -maxlenltr 7000 -mintsd 4 -maxtsd 6 \  
  -motif TGCA -motifmis 1 > genome.harvest.scn
```

Input from *LTR_FINDER*:

```
ltr_finder -D 15000 -d 1000 -L 7000 -l 100 \  
  -p 20 -C -M 0.9 genome.fa > genome.finder.scn
```

Input from *MGEScan_LTR* (a modified version obtained from DAWGPAWS (11)):

```
perl find_ltr_DAWGPAWS.pl \  
  -seq=genome.fa \  
  -min-ltr=100 -max-ltr=7000 -min_iden=90
```

The `-nonTGCA` input can be obtained without specifying `-motif TGCA -motifmis 1` in *LTRharvest*:

```
gt ltrharvest \  
  -index genome.fa \  
  -similar 90 -vic 10 -seed 20 -seqids yes \  
  -minlenltr 100 -maxlenltr 7000 -mintsd 4 -maxtsd 6 \  
  > genome.harvest.nonTGCA.scn
```

6 Outputs

The output of *LTR_retriever* includes:

- A summary table for the identified intact LTR-RTs with coordinate and structural information

(***.pass.list**)

- A non-redundant LTR-RT library (exemplar) in the **FASTA** format (***.LTRlib.fa**)
- A **GFF3** format file for all intact LTR-RTs (***.pass.list.gff3**)
- A **GFF** format file for the whole-genome LTR-RT annotation (***.gff**)

Example of an intact LTR-RT list:

```
#LTR_loc Category Motif TSD 5'_TSD 3'_TSD Internal Similarity Strand Family
Superfamily Insertion_Time
Chr10:10211053..10223177 pass motif:TGCC TSD:GGTGG 10211048..10211052
10223178..10223182 IN:10211491..10222739 0.9794 - Gypsy LTR 1030000
Chr10:11328988..11335399 pass motif:TGCA TSD:CAGTC 11328983..11328987
11335400..11335404 IN:11329472..11334915 0.9587 - Copia LTR 2065000
Chr2:16844554..16849613 pass motif:TGCA TSD:GCATG 16844549..16844553
16849614..16849618 IN:16845219..16848945 0.9416 ? unknown NA 2920000
Chr2:17280296..17282788 pass motif:TGCA TSD:TATAC 17280291..17280295
17282789..17282793 IN:17280474..17282616 0.9497 + unknown LTR 2515000
Chr2:17891339..17904598 pass motif:TGCA TSD:CCCTC 17891334..17891338
17904599..17904603 IN:17892615..17903320 0.9867 ? Gypsy LTR 665000
```

Example of an LTR library:

```
>Chr10:1057194..1057414_LTR#LTR/Copia
TGTTGGCGAACGGCTTCGTCAGACTCTCGCGGCGCGCTCCACGCGCACGACGCGCACCC
CGCGCACGACGCGCAGCGCTCCTCGCTCCGCTCGCACCGCTGCACGTCCGTTAGACCAG
GGGATTAGTTAGGCCAGGCAACTCCCAAGCCTTGTGTACATGTATAAATGTAAGCTC
CATTGATCAATGAAAGTTACGGTTGATCCAAATCTCCTTCTACA
>Chr10:12110230..12110468_LTR#LTR/unknown
TGTCATGGGCTTTGGGCCGGGAGTCCTAGGCCCATGAGATAGAATTAGGGTTTGTTAGG
ATTAGATAAGGTTTGTAGGATTAGATTAAGTAGCCCTCCATCTATATAAGGAGGGATC
CTATCCAGGTCAGTTAGGCATTAGATCAATATTTATCTTAGTGCCCATCGGCCTGCCT
TCTCAGTGCGACGGAGAGCGTCGCGCCGTTAGGTTGAGGACCGTATTCTTGTTCGTG
ACA
>Chr10:10063621..10068275_INT#LTR/Gypsy
AATCCACCCCCCTTACAAGAATTCGTCCCCGAGATTCGAGGAGGCTAGCATGAAGATA
...
CTACATGCCGCTAGCGGATCCTGCAGTCTTCGGAGCTTCGGCAAACAATTGGCGACAT
CTTCTTCTTCGCAAAGCTGACCATCTAGTACTAGTTGAAAATCCGAAGGAGGAAGAAGA
CAATAAACATTTTGCAAAT
```

7 Usage

7.1 *LTR_retriever* is called as follows:

```
LTR_retriever -genome genomefile -inharvest LTRharvest_input [options]
```

where `-genome` specifies the genome sequence and is also used as the root file name of outputs; the `-inharvest` parameter specifies the LTR-RT candidate file obtained from *LTRharvest*. Multiple candidate sources can be used (see **Table 1**).

For example,

User provides only one candidate source:

e.g. 1

```
LTR_retriever -genome genome.fa -infinder genome.finder.scn
```

e.g. 2

```
LTR_retriever -genome genome.fa -inharvest genome.harvest.scn
```

e.g. 3

```
LTR_retriever -genome genome.fa -inmgescan genome.MGEScan.scn
```

User provides multiple candidate sources:

e.g. 4

```
LTR_retriever \  
-genome genome.fa \  
-inharvest genome.harvest.scn \  
-infinder genome.finder.scn
```

e.g. 5

```
LTR_retriever \  
-genome genome.fa \  
-inharvest genome.harvest.scn \  
-infinder genome.finder.scn \  
-inmgescan genome.MGEScan.scn
```


To recover non-canonical LTR-RTs, you may use the `-nonTGCA` option to provide extra candidates along with other input(s) (either one source or multiple sources).

e.g. 6

```
LTR_retriever \
  -genome genome.fa \
  -inharvest genome.harvest.scn \
  -nonTGCA genome.harvest.nmtf.scn
```

e.g. 7

```
LTR_retriever \
  -genome genome.fa \
  -infinder genome.finder.scn \
  -inharvest genome.harvest.scn \
  -inmgescan genome.MGEScan.scn \
  -nonTGCA genome.harvest.nonTGCA.scn
```

7.2 WARNINGS

LTR_retriever will alter sequence names longer than 20 characters to fit the naming requirement of *RepeatMasker*. **Please backup your original genome file before using *LTR_retriever*.**

LTR_retriever can take multiple sources as inputs for one single run, but running multiple instances of *LTR_retriever* in the same folder at the same time may cause errors.

7.3 An overview of all parameters

Table 1. All parameters for *LTR_retriever*.

Input options	
<code>-genome</code> [FASTA File]	specify the genome sequence file (in FASTA format)
<code>-inharvest</code> [File]	LTR-RT candidates obtained from the screen output of <i>LTRharvest</i> with <code>-motif TGCA</code> parameters
<code>-infinder</code> [File]	LTR-RT candidates obtained from the screen output of <i>LTR_FINDER</i>
<code>-inmgescan</code> [File]	LTR-RT candidates obtained from the output of <i>MGEScan_LTR</i> (the .ltrloc file)
<code>-nonTGCA</code> [File]	Non-canonical LTR-RT candidates obtained from the screen output of <i>LTRharvest</i> with default parameters
Output options	
<code>-verbose</code>	retain intermediate outputs (developer mode)

-noanno	disable whole genome LTR-RT annotation (no GFF output)
Filter options	
-misschar [CHR]	specify the character for ambiguous sequences in the genome (default N)
-Nscreen	disable filtering ambiguous sequence in LTR-RT candidates (default enable (by not specifying this flag))
-missmax [INT]	specify the maximum number of ambiguous bp allowed in an LTR-RT candidate (default 10)
-misrate [0-1]	specify the maximum percentage of ambiguous length bp allowed in an LTR-RT candidate (default 0.8)
-minlen [INT]	specify the minimum length (bp) of the LTR region (default 100)
-max_ratio [FLOAT]	specify the maximum length ratio of the internal region length over the LTR region length (default 50)
-minscore [INT]	specify the minimum alignment length (INT/2) to identify and filter out tandem repeats in an LTR-RT candidate (default 1000)
-flankmiss [1-60]	specify the maximum gap length (bp) allowed in 60bp-flanking sequences (default 25), smaller number indicates higher stringency
-flanksim [0-100]	specify the minimum percentage of identity for flanking sequence alignment (default 60)
-flankaln [0-1]	specify the maximum alignment portion allowed for 60bp-flanking sequences (default 0.6)
-motif [[STRING]]	specify a list of (known) motifs in square brackets as the prior knowledge to search for non-canonical LTR-RTs (default -motif [TCCA TGCT TACA TACT TGGA TATA TGTA TGCA])
-notrunc	Discard sequence information from truncated LTR-RTs and nested LTR-RTs (will dampen sensitivity) (default retain (not specifying this flag))
-procovTE [0-1]	specify the maximum portion of an LTR-RT candidate allowed for cumulated alignments to the DNA TE database and the LINE database (default 0.7)
-procovPL [0-1]	specify the maximum portion of an LTR-RT candidate allowed for cumulated alignments to the plant protein database (default 0.7)
-prolensig [INT]	specify the minimum alignment length (bp) to be counted for LINE/DNA transposase/plant protein alignment (default 90)
Library options	
-blastclust [[STRING]]	specify <i>blastclust</i> parameters in square brackets (default -blastclust [-L .9 -b T -S 80]). By triggering this tag without specifying any parameters (-blastclust), <i>blastclust</i> will be turned on (default off) with default parameters. <i>blastclust</i> settings refer to http://www.ncbi.nlm.nih.gov/Web/Newsltr/Spring04/blastlab.html
-cdhit	specify <i>cd-hit-est</i> parameters in square brackets (default -cdhit

[[STRING]]	[-c 0.8 -G 0.8 -s 0.9 -T 20 -aL 0.9 -aS 0.9]). By triggering this tag without specifying any parameters, <i>cd-hit-est</i> will be turned on (default on) with default parameters. <i>cd-hit-est</i> settings refer to http://weizhongli-lab.org/cd-hit/wiki/doku.php?id=cd-hit_user_guide
-linelib [FASTA File]	specify a custom LINE transposase database for LINE TE exclusion (default LTR_retriever/database/Tpases020812LINE)
-dnalib [FASTA File]	specify a custom DNA TE transposase database for DNA TE exclusion (default LTR_retriever/database/Tpases020812DNA)
-plantprolib [FASTA File]	specify a custom plant protein database for protein coding sequence exclusion (default LTR_retriever/database/alluniRefprexp082813)
-TEhmm [Pfam File]	specify a custom Pfam database for TE identification (default LTR_retriever/database/TEfam.hmm)
Miscellaneous	
-u [FLOAT]	specify the neutral mutation rate of the target species (per bp per year) (default 1.3e-8 (from rice (12)))
-threads [INT]	specify the number of threads (\leq total available threads, default 4)
--help (-h)	display the help information

8 Benchmarks

Run time of LTR_retriever is roughly proportional to total candidate number (input) as shown in **Table 2**. The size and the LTR-RT fraction of a genome together determine the number of LTR-RT candidates identified by prediction programs.

Table 2. Benchmark of LTR_retriever in model genomes.

	Arabidopsis	Drosophila	Rice (MSU v7)	Sacred lotus	Maize (B73 v4)
Genome size (Mb)	120	144	374	708	2,134
Raw candidates	2335	2642	5436	12011	114048
Intact LTR-RT	232	517	2129	918	43227
Fraction masked	7.4%	12.4%	25.3%	29.6%	70.1%
Library entry	233	359	1529	1467	12360
Run time (-threads 20)*	10 min	10 min	42 min	2.1 hr	94.9 hr

*not including the time of whole-genome LTR-RT annotation.

9 Reusable Scripts

LTR_retriever was built on several flexible Perl scripts which are useful for other research purposes. This section describes some of the most useful ones based on the developer's experience. These reusable scripts include many more others are located in **LTR_retriever/bin/**

Script: *annotate_gff.pl*

Description: Annotate the **GFF** file generated by *RepeatMasker* using the LTR library generated by *LTR_retriever*.

Usage: perl annotate_gff.pl **genome_LTRlib.fa genome.gff** >
genome.anno.gff

Options: None.

Script: *call_seq_by_list.pl*

Description: Extract sequence from the user provided genome (**FASTA** format) using a file containing a list of coordinates (one line each) in the MSU locus format (e.g., target1 Chr01:10000..11000). The script can output sequence in its minus direction. If the locus coordinate is written backward (e.g., Chr1:2000..1000), it would be treated as a negative strand request.

Usage: perl call_seq_by_list.pl **MSU_format_list -C genome.fa** [options]

Options:

itself (default)	extract the sequence specified by the coordinate
up_[INT]	extract the sequence [INT] bp upstream of the specified coordinate
down_[INT]	extract the sequence [INT] bp downstream of the specified coordinate
-rmvoid	if this is triggered (default off), skip printing any void sequence (e.g., no such sequence in the genome)
-ex	if this is triggered (default off), execute the exclude function instead of the extract function
-purge [0/1]	use with -ex, if this is triggered (1, default 0), sequence specified by the list file will be excluded from the provided FASTA file
-cov [0-1]	use with -ex, sequence with removal length longer than the specified portion (default 0.7) will be entirely excluded

Script: *purger.pl*

Description: Purge the provided FASTA file with BLAST alignment output.

Usage: perl purger.pl -blast **blast_outfmt6 -seq FASTA** [options]

Options:

-eval [FLOAT]	for BLAST hits, e-values (e.g. 1e-10) lower than this cutoff (default 0.001) is considered a real alignment
-len [INT]	length of alignment hits (bp, default 90) to be considered as a real alignment
-cov [0-1]	if the excluded portion of a sequence exceeds the specified value (default 1), discard the entire sequence
-purge [0/1]	if this is triggered (1, default 1), sequence regions identified by BLAST will be excluded from the provided FASTA file. If this is not triggered, the entire sequence will be excluded if it achieves the cutoff defined in “-cov”. Otherwise the matched portion will be retained.

Script: *PacBio_processor.pl*

Description: Convert **fastq** files (e.g., PacBio reads) into **FASTA** files with simple filtering options.

Usage: perl *PacBio_processor.pl* **PacBio.fastq** > **PacBio.fasta**

Options (modified in the script):

minLength	minimal read length (bp) (default 500)
maxLength	maximal read length (bp) (default 50000)
minRQ	minimal read quality (default 0.8)

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