mRNA annotation & assembly pipeline (`MAAP`) - Manual

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Introduction

MAAP is a transcriptome assembly and mRNA annotation pipeline, which utilizies external and newly developed software components. Starting with RNA-seq data and a reference transcriptome, **MAAP** basically performs 3 steps: de novo transcript assembly (Trinity), gene symbol assignment (best bidirectional blastn hit) and sequence feature annotation (multiple sequence alignment, GENSCAN, …). Additional characteristics are scaffolding of fragmented transcript and splitting/clipping of misassembled transcripts. Further details: [Publication]

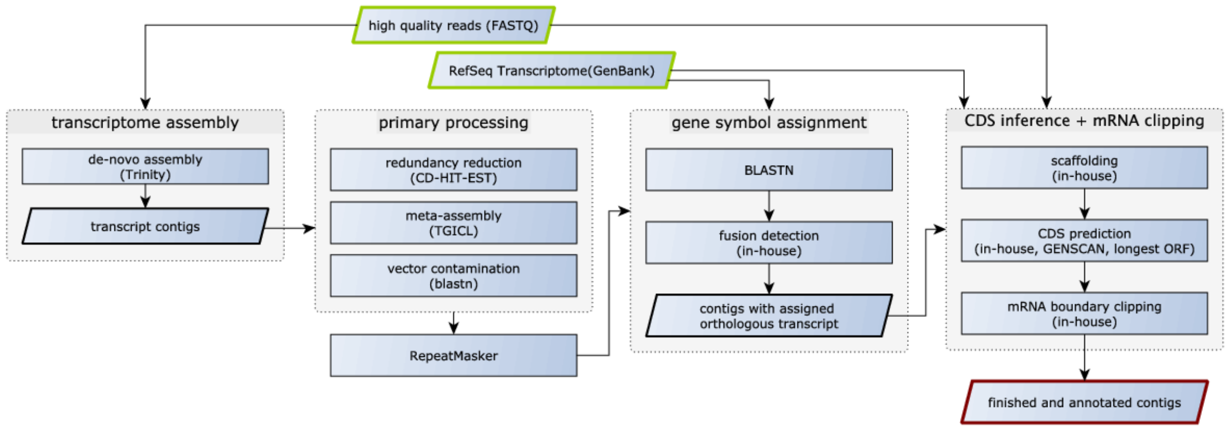

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Figure 1: Pipeline

Input

Essentially, all you need is a reference transcriptome in GenBank format and RNA-seq data in FastQ format. You can also provide **MAAP** with orthologs to your reference transcripts (see 'Configuration' for further information). The additional orthologous coding sequences are used for CDS inference.

Dependencies

General

- Linux (should work with MacOS, but untested)
- GNU parallel
- R (3.0.3): CRAN packages: `plyr`, `ggplot2`, `reshape`, `gridExtra`
- Bioconductor packages: `KEGG.db`, `GO.db`, `annotation`
- Perl (5.10.0; all modules available at CPAN): `BioPerl`, `Parallel::ForkManager`, `Bio::Range`, `Set::Intspan`

Bioinformatic Software

Exclude CD-HIT-EST and TGICL if primary preprocessing is not intended. Also, set `REPEAT := 0` in order to skip repeat masking (not recommendend).

- mandatory: Trinity, WU-BLAST, EMBOSS, MAFFT, GENSCAN, bamtools, bowtie2, samtools
- optional: CD-HIT-EST, TGICL, RepeatMasker, SGE/OGE (qsub)

Installation

Installing **MAAP** is quick and easy. Download and unpack this repository and make sure you have permission to execute **MAAP**. You can add **MAAP** to your $PATH or create a symlink to **MAAP** in one of the directories in $PATH.

Here is a suggest workflow, which adds *MAAP* to your `$PATH`:

```
#!bash
unzip MAAP.zip
cd MAAP/bin/
chmod a+x MAAP
PATH=$(pwd):$PATH
export PATH
```

Run

Make sure all mandatory parameters are specified in the configuration file (see Configuration section). Then, call **MAAP** with the appropiate configuration file.

```
        MAAP configuration_file
```

That's all. In case of aborts, consult logfiles and remove incomplete results. Rerunning the above command will complete remaining tasks.

Same as above, but shows all called processes.

```
        MAAP configuration_file verbose
```

Start from scratch (removes all created files beforehand).

```
        MAAP configuration_file scratch
```

MAAP uses GNU make as a backbone. Parameters other than `verbose` and `scratch` (and `full-cleanup`, `cleanup`) are forwarded to make. For example, the following lists all tasks without executing them.

```
        MAAP configuration_file -n
```

Cleanup

MAAP creates a lot of intermediate files. See "output files" for further information about each file. We provide to two cleaning methods:

```
MAAP configuration_file full-cleanup
```

This will keep all important files: * `sequences-mRNA.fasta`, * `sequences-CDS.fasta`, * `transcript_catalogue.gbk`, * `summary*` * `tables/*`

```
MAAP configuration_file cleanup
```

additionally keeps: * `transcripts/`, * `trinity/`

Configuration

Take a look at and try to run the provided example file in `PATH_TO_MAAP/example/testing.conf` before running **MAAP** on your own data set.

This also serves as a template for your custom configuration.

mandatory variables

The following depends mostly on your `$PATH` variable. Specify path to **directories**(!) of executables for each program that is not in your `$PATH`. Otherwise, remove line or leave empty.

```
PATH_BAMTOOLS     :=
PATH_BOWTIE       :=
PATH_CD_HIT_EST   := /home/user/src/cd-hit/
PATH_EMBOSS       := /home/user/src/EMBOSS/bin/
PATH_GENSCAN      :=
PATH_MAFFT        := /home/user/src/mafft/
PATH_PERL         :=
PATH_REPEATMASKER :=
PATH_RSCRIPT      := /home/user/src/R/bin/
PATH_SAMTOOLS     :=
PATH_TGICL        :=
PATH_TRINITY      :=
PATH_WUBLAST      :=
PATH_XDFORMAT     :=
PATH_GENSCAN_MAT  := (point to actual file)
```

Store intermediate and final files in specified location. Make sure that enough space is available to store intermediate output of trinity, blast results, read alignments, ...).

```
OUTPUT_DIR := /data/output
```

Input reads in fastq format. In case of paired end data, indicate elements of pair by "R1" and "R2" in filename (Example: `sampleA_R1.fq`, `sampleA_R2.fq`). All files must be in the same format (one of fastq, fasta, gzipped).

```
READ_DIR := /data/reads/
```

Reference transcriptome in GenBank format as provided by NCBI: `http://ftp.ncbi.nlm.nih.gov/genomes`

```
REF_TRANSCRIPTOME := /data/human.gb
```

Specify taxonomy id of species to assemble. MAAP connects to NCBI (once) to fetch necessary species information.

```
SPEC_TAXID := 458603
```

We use genome wide annotation packages from Bioconductor to assign functional annotation to the resulting transcript catalogue. Provide annotation package corresponding to your reference species.

```
OPT_ANNOTATION := org.Hs.eg.db
```

optional

If you already have extracted mRNA and CDS sequences in fastA format, provide them to **MAAP**. Additionally, you can add a repeat (soft) masked fastA of your reference sequence in order to skip RepeatMasking step.

```
REF_TRANSCRIPTOME_FASTA              := /data/human_mRNA.fa
REF_TRANSCRIPTOME_FASTA_MASKED       := /data/human_mRNA.fa.masked
REF_TRANSCRIPTOME_FASTA_CDS          := /data/human_cds.fa
REF_TRANSCRIPTOME_FASTA_CDS_MASKED := /data/human_cds.fa.masked
```

CDS inference is based on the coding sequence of the orthologous reference transcript. You can extend the number of orthologs used to infere the appropriate CDS by providing a table with mappings between orthologous transcript from different species. The first column must contain accession of the reference transcript. Add one column for each species you want to use and use 'NA' to indicate unknown orthologs. Additionally, specify taxonomy ID of each species in the first line (starting with #, tab separated). Keep in mind, that we perform a multiple sequence alignments with all coding sequences. Therefore, the number of species used will have an influence on runtime. Additionally, you must provide a fasta file containing all coding sequences mentioned in table (`ORTHOLOG_FASTA`).

```
ORTHOLOG_TABLE := /data/ortholog_table.csv
ORTHOLOG_FASTA := /data/ortholog_cds.fa
```

Example content ORTHOLOG_TABLE (also, take a look at exampe/ortholog_table.csv)

```
#9606   10090   10116   9615
NM_130786       NM_001081067    NM_022258       NA
NM_001198819    NM_001081074    NM_133400       XM_534776
NM_001198818    NM_001081074    NM_133400       XM_534776
```

We keep a note in GenBank output about the ortholog used to annotated CDS. Please specify the order of columns (0-based) based on `ORTHOLOG_TABLE` to indicate your prefered order of species in case of multiple equally valid coding regions. Example:

```
SPECIES_ORDER := 0,2,1
```

Specify preprocessing steps you want to apply to the raw trinity assembly (space separated list) in prefered order. Possible steps are: `cd-hit` and `tgicl`.

```
ASSEMBLY_PREPROCESS :=
```

Soft masks repeats in assembly and reference. Set to 0 if you want to skip repeat masking.

```
REPEAT := 1
```

Software parameter

!Consult manual for external software!

Number of cpus. This will be used for any software which runs in parallel.

```
OPT_CPUS := 2
```

If SGE is available (qsub), it will be used for blast jobs. Specify number of jobs.

```
OPT_MAX_SGE := 20
```

Trinity

Single end (s) or paired end (pe) reads?

```
    OPT_READTYPE := s
```

Consult trinity manual.

Added automatically: `--no_cleanup`

```
    OPT_TRINITY   := --JM 10G --seqType fa
    OPT_BUTTERFLY :=
```

RepeatMasker

Repeat masking reference/assembly. We will add `-xsmall -par OPT_CPUS` automatically.

```
    OPT_REPEAT_REF_TRANSCRIPTOME := -species human -engine ncbi
    OPT_REPEAT_ASSEMBLY          := -species human -engine ncbi
```

CD-HIT-EST

Added automatically: `-T OPT_CPUS`

```
    OPT_CD_HIT_EST :=
```

TGICL

Added automatically: `-c OPT_CPUS`

```
    OPT_TGICL :=
```

misassembled contigs

Used to detect fusion transcript. Specify maximum overlap (`-max-overlap`) between CDS regions (specifically: blast hits by coding sequences of reference transcriptome), minimum length of alignment (`-min-frac-size`), identity (`min-identity`) and coverage (`min-coverage`) thresholds.

```
   OPT_FUSION := -max-overlap 5.0 -min-frac-size 200 -min-identity 70.0 -min-coverag
```

WU-BLAST

Added automatically: `-wordmask=seg lcmask -topcomboN 3 -cpus 1`

```
    OPT_WUBLAST_BLASTN :=
```

SBH requiremnts

Specify minimum required identity and coverage to consider hit as SBH.

```
    OPT_SBH := -identity=70.0 -coverage=30.0
```

Scaffolding

Specify minimum required identity and contig coverage of blast hit to consider contig as possible scaffolding fragment.

```
    OPT_FRAGMENTS   := -identity 70.0 -query-coverage 90.0
```

Specify minimum overlap between fragments in alignment to apply filtering rules (example: keeps sequence with higher similarity to reference if fragments differ over 98% in overlap, if overlap exceed 66% of contig length)

```
    OPT_SCAFFOLDING := -fragment-overlap 66.0 -fragment-identity 98.0
```

CDS prediction

Add '-predictions' if you don't want to use predicted coding sequences (XM Accessions) for CDS inference. Don't use that if your reference contains "XM" Accessions [TODO].

```
    OPT_PREDICTCDS := -predictions
```

Output files

important files

File	Description
transcript_catalogue.gbk *	GenBank file describing **all annotated sequences**.
sequences-CDS.fa	Fasta with **coding sequences**.

File	Description
sequences-mRNA.fa	Fasta with **transcript sequences** (w/o introns; clipped ends).
assembly.fa	Repeat masked **trinity assembly**.
summary.pdf	General overview of transcript catalogue|
tables/summary.csv	Table containing summary for each annotated transcript.
tables/cds.csv	CDS coordinates (1-based) on sequences-mRNA.fa.
tables/annotation.csv	Contig annotation (contig, refseq, strand, symbol).
tables/overview.csv	Transcript catalogue details .
tables/transcriptome_statistic.csv	Table containing descriptive statistics about transcript catalogoue.

*mRNA feature instead of 'gene' feature to limit mRNA boundaries in case of misassembled contigs

functional annotations (based on reference)

Table containing GO Terms associated with each annotated transcript. Also, overview of covered GO Terms and genes in total (genes_per_ontology) and in more detail (genes_per_path).

```
tables/gene_ontology.csv
tables/gene_ontology_genes_per_ontology.csv
tables/gene_ontology_genes_per_path.csv
```

Same as above, but for KEGG Pathways.

```
tables/kegg.csv
tables/kegg_covered.csv
tables/kegg_genes_per_path.csv
```

intermediate output

trinity/

Trinity output (including intermediates).

transcripts/

Running **MAAP** creates a lot intermediate output which might come in handy in downstream analysis. Each transcript assignment is stored in a separate directory in

```
transcripts/
```

with the naming pattern according to assigned ortholog.

```
transcripts/SYMBOL_ACCESSION/
```

This directory includes the following files:

Result in GenBank format.

```
_final.gbk
```

Raw GENSCAN output.

```
CDS_genscan.txt
```

Assignment of transcript accession to GENSCAN prediction based on blast hits.

```
CDS_genscan_annotated.txt
```

Multiple sequence alignment with orth. species requested in `ORTHOLOG_TABLE`

```
CDS_alignment.aln
```

BLAST databases for reference and assembly.

```
db/
```

BLAST results including average for each HSP-group (`avg_*`) and best hit per query (`best_*`).

```
blast/raw_*
blast/avg_*
blast/best_*
```