miRNAture

Release 1.0.0

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CONTENTS:

Computational detection of microRNA candidates.

CHAPTER

ONE

INTRODUCTION

MicroRNAs (miRNAs) have been characterized as an important regulators in almost all animals and plants, as well as in unicellular eukaryotes [11]. Since their discovery in 1993 [10] and subsequently in their recognition as a biological entity later from 2000 [14], microRNAs were identified as key regulators on the temporal control of heterochronic genes on the nematode *Caenorhabditis elegans* and subsequently in another metazoan species [12]. This recognition were complemented by a complete characterization of their typical features as: a stem-loop structure, well-conservation over multiple metazoan clades and typical expression patterns as an isolated locus or co-expression of polycistronic miRNA transcripts [7][8][9][14].

Now, metazoan miRNAs have been recognized as a conserved group of short ncRNAs, typically ~ 22 nt, with important roles as post-transcriptional regulators of the gene expression affecting a sizeable number of mRNAs and expressed in all developmental process and diseases [2]. Their canonical biogenesis starts in the form of primary precursors (pri-miRNAs) transcribed from long non-coding RNAs or protein-coding transcripts, mostly from introns [17]. Later, derived from hairpin-like precursors excised in the nucleus (pre-miRNAs), their acting form is subsequently further processed as miRNA/miRNA* duplexes on the cytoplasm and incorporated into the RISC complex. Target specificity is achieved by complementarity between the miR and mRNA sequence. (see more details in [2]).

Current classification of annotated miRNAs into families are available in miRBase[1] and mirGeneDB[2] databases. As an example, the human genome reported 1984 miRNA precursors in the miRBase v.22.1 [6] and the corresponding mature products were estimated ~2300 [1]. Focusing on the *confidently canonical* miRNAs reported in [4], the number of miRNAs is 519. Those differences are explained on the basis of multiple *miRNA* detection methods as well as the intrinsic definitions to define a *canonical miRNA*.

Despite the small size of the precursors (80-100 nt), sequence comparison methods are able to detected them, due a high level of sequence conservation [13]. In one hand, it is important to point that the use of blast-based homology searches alone tend to produce false positives that require extensive curation, which relies on properties obtained from miRNAs [15]. On the other hand, the inclusion of the consensus structure complements the homology search methods, for example using covariance models (CMs) [3][5]. The accuracy and sensitivity of CMs depends critically on the sequence alignment and the annotated consensus used to build the model. Those observations call for an integrated workflow to perform homology search and to evaluate their results in a consistent manner.

In this computational approach, focused on the *canonical* miRNAs processed by Drosha and Dicer, we improve on ideas from MIRfix [16] and integrate it with homology search. `miRNAture` is used to identify and annotate homologs of metazoan microRNAs in a homology-based setting.

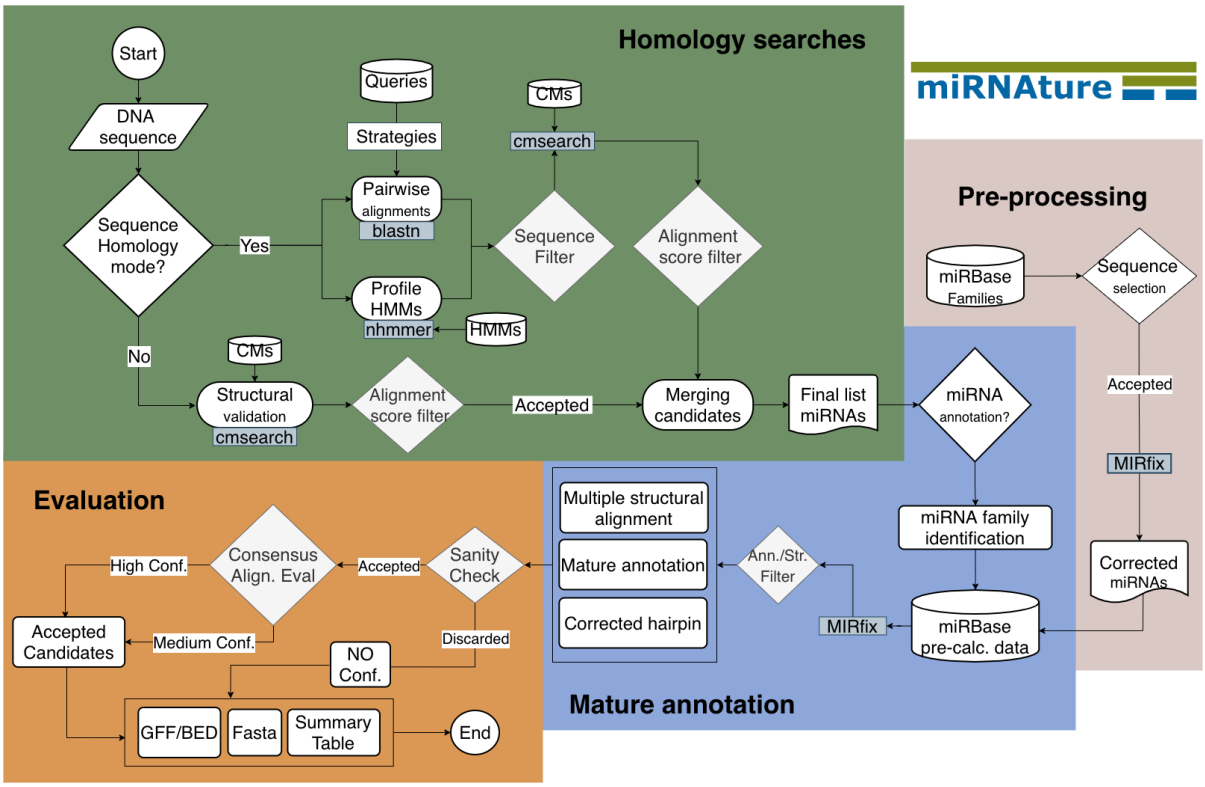
[1] https://www.mirbase.org/
[2] https://www.mirgenedb.org/

CHAPTER

TWO

STRATEGY

Current approaches to computational miRNA detection relies on homology relationships or detection of hairpin-loop candidates with lower folding energy. A complete set of tools to automatize this task have been assembled on `miRNAture`. This current approach combines two different sequence-homology modes, using `blast` or `HMMer`, and a secondary structure validation step, performed by the `INFERNAL` package. Combination of different strategies and the modification of default covariance models, let `miRNAture` report not only the homology relationships, but also define positions of *mature* sequences and the correct miRNA annotation, supported by multiple family specific alignments. Current workflow is depicted as follows:



CHAPTER

THREE

INSTALLATION

The easiest way to install **miRNAture** is through *conda*. To do so, please first install Conda[3].

To speed up installation of dependencies and packages we suggest to use mamba[4], for this just run:

```
conda install mamba -c conda-forge
```

You can use *mamba* as drop-in replacement for *conda* by simply replacing the call to *conda* with a call to *mamba*.

Install via Conda

To install **miRNAture** from *conda* in a specific *mirnature* environment simply run:

```
mamba create -n mirnature mirnature
```

if *mamba* is available, else run:

```
conda create -n mirnature mirnature
```

Manual install, resolve dependencies via Conda

Create a *mirnature conda* environment with the file *miRNAture.yml*:

```
mamba env create -n mirnature -f miRNAture.yml
```

Activate the environment containing all dependencies:

```
conda activate mirnature
```

followed by the manual steps:

```
perl Build.PL
./Build
./Build test
./Build install
```

which will install **miRNAture** in the *mirnature conda* environment.

[3] https://docs.conda.io/projects/conda/en/latest/user-guide/install/
[4] https://github.com/mamba-org/mamba

3.1 Tutorial

3.1.1 Annotating (some) coelacanth miRNAs

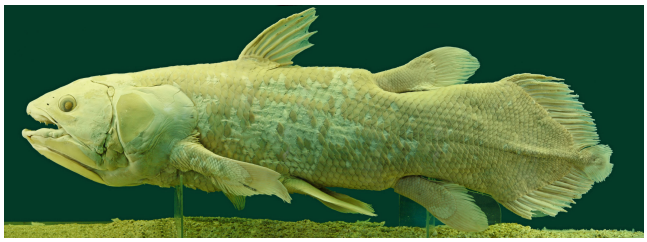


Fig. 1: *Latimeria chalumnae*. Source: Alberto Fernandez Fernandez / CC BY-SA

Throught this step-by-step tutorial you could make use of key options from `miRNAture` to annotate the *bona fide* miRNA complement on selected contigs from the coelacanth (*Latimeria chalumnae*) genome, based on the current miRNA annotation, retrieved from `Ensembl` release 100. The following table shows the features from selected contigs that composed the fasta file with subject sequences:

Contig	Length (Mb)	Numb. miRNAs
JH126571.1	5.98145	5
JH126620.1	3.03251	8
AFYH01291077.1	0.00106	1

Your task will be the identification of homologous miRNAs on the described contigs. To perform this taks, `miRNAture` makes use of pairwise alignments with `blastn` and the use of hidden Markov models using `nhmmer`. To the validation steps rounds of structural alignments, using `cmsearch`, would be applied. The final validation step, will be performed by `MIRfix` in order to annotate the correct positions of candidate mature regions along the detected hairpin sequence.

As you can imagine, there would be created both, a high number of input and output files and classification rules to parse and select the candidate miRNA regions. But, do not worry too much about this! life is too short to perform all of this task by hand! and `miRNAture` will help to perform all the heavy and painstaking work.

Folder structure

The folder tree on miRNAture looks like:

```
$ tree -L 1 miRNAture/
miRNAture/
├── Code
├── LICENSE
├── Manuscript
└── README.md
```

Out target folder is located on `Code/Tutorial`:

```
$ cd Code/Tutorial
$ tree -L2 .
Tutorial
├── Code
│   └── run_miRNAture.sh
```

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```
├── Data
│   ├── annotated_miRNAs_latch.gff3
│   ├── latimeria_chalumnae_genome.fa
│   ├── Latimeria_chalumnae.LatCha1.100.gff3
│   └── QueriesToTest
└── Results
```

The `Tutorial` folder is composed by the subfolders: `Code/`, where all the necessary scripts to run `miRNAture` are located. `Data/` keeps the described genome from coelacanth in a multi-fasta format in `latimeria_chalumnae_genome.fa`. Another key folder is `QueriesToTest/`, where the miRNAs from 11 chordates were provided to serve as query sequences. Detailed list of query species and their correspondent files are described on `QueriesToTest/queries_description.txt`. The set of files inside `Data/` corresponds to the current and filtered miRNA annotation of coelacanth retrieved from `Ensembl` release 100: `Latimeria_chalumnae.LatCha1.100.gff3` and `annotated_miRNAs_latch.gff3` in `GFF3` format, respectively. The last `Results` folder will conserve all the output files generated by `miRNAture`.

Input files

As described earlier, to run `miRNAture` just go directly to `Code/`, located on the `Tutorial/` folder:

```
$ cd Code/
$ ls -ls
4 -rwxr-xr-x 1 cristian students 598 Jul  8 18:47 run_miRNAture.sh
```

As you noted, exists a bash file inside this folder which will organize all our code to run `miRNAture`. This way is preferred, if you think about increase the reproducibility of your computational experiments. Looking in detail this code will give you a general idea to run `miRNAture`:

```
#!/bin/bash

# Declare input folder
current=$( pwd )

# Step 1: Activate conda environment
conda activate miRNAture

# Step 2: Input files/folders
specie_tag="Latch"
specie_genome="$current/../Data/latimeria_chalumnae_genome.fa"
specie_name="Latimeria_chalumnnae"
workdir="$current/../Results"
mirfix_path="/homes/biertank/cristian/Projects/MIRfix/scripts/MIRfix.py"
mode="Blast,HMM,Infernal,Final"
strategy="1,2,3,4,9,10,ALL"
blastQueriesFolder="$current/../Data/QueriesToTest"

# Step 3: Running miRNAture
cd $current/../../../Code/

# Step 3.1: Run homology-searches:
./miRNAture -stage homology -speG $specie_genome -speN $specie_name \
 -speT $specie_tag -w $workdir -mfx $mirfix_path -m $mode -pe 0 \
 -str $strategy -blastq $blastQueriesFolder

#Step 3.2: Validate miRNAs annotating their mature sequences:
```

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```
./miRNAture -stage validation -speG $specie_genome -speN $specie_name \
 -speT $specie_tag -w $workdir -mfx $mirfix_path -m $mode -pe 0 \
 -str $strategy -blastq $blastQueriesFolder
```

The last script shows three steps that are required to run `miRNAture`:

1. Activate the `conda` environment called miRNAture. The installation and activation of this environment is required previously to run `miRNAture`. All the dependences are described on the file `miRNAture.yml`, located on the `miRNAture/Code/` folder.
2. Declare the name of input and output locations. `miRNAture` detects different flags with their correspondent values. The basic configuration is composed by:
   - Specie genome: Current target sequence
   - Specie name: Scientific name of the specie which belongs the subject sequence(s).
   - Specie tag: Tag of the specie name, suggested one takes the first two letters from the Genera joined with the first two from the specie (i.e Homo sapiens = hosa, Didemnum vexillum = dive, Latimeria chalumnae = lach).
   - Working directory: Output directory, final path of `miRNAture` results.
   - MIRfix path: path of MIRfix on your system.
   - Running mode: Select at least one, or any combination of the miRNA search strategies between: `Blast`, `HMM` or/and `Infernal`. `At the same time, to merge the complete results from those homology search modes, write at the end ``Final`.
   - Blast strategies: Write the numbers of desired `blastn` strategies. Possible strategies are: `1,2,3,4,5,6`. At the same time, to merge all results put at the end `ALL`.
   - Path of `blastn` queries: Declare the path of annotated query sequences of miRNAs. In this case is enough to indicate the folder name.
3. Run `miRNAture`. Setup all the command line options based on the described input files on step 2. The list of complete flags can be found at:

```
$ ./miRNAture --help
Usage:
./miRNAture [-options]

Options:
-help       print this documentation

-man        Prints the manual page and exits.

-stage      Selects the running mode of miRNAture. The options are:
            'homology', 'validation' or 'complete'.

-speG       path of target genome or genomic sequence to be analyzed

-speN       Specie or sequence source's scientific name. The format must
            be: Genera_specie, separated by '_'.

-speT       Tag of the specie, sequence or project. Just for future
            reference.

-w          Path of working directory
```

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```
-mfx        Path of the MIRfix
            <https://github.com/Bierinformatik/MIRfix> program:
            "MIRfix.py"

-m          Homology search modes: Blast,HMM,Infernal and Final. It is
            possible to perform individual analysis, but it is always
            desirable include the Final option.

-str        This flag is blast specific. It corresponds to the selected
            blast strategies used to search miRNAs. It might be
            indicated along with -m Blast or in case you refer it in
            your selected mode.

-blstq      Path of blast queries sequences in fasta format to be
            searched on the subject sequence.
```

Searching miRNAs

The most important step will be performed! Based on the last configurations on the script `run_miRNAture.sh`, `miRNAture` will be executed on the designed coelacanth sequences. The idea is to perform independently each of the stages, *homology-searches* and *detection of mature*, for demonstrative purposes. In case that you require to run the complete pipeline, just adjust the parameter **-stage** to the *complete* option.

Homology search

As mentioned, we are going to execute the *homology-search* stage. To activate this stage in `miRNAture` please verify the flag value to be **-stage homology**. In brief, our *target* coelacanth sequences would be annotated using a set of miRNA *queries* that belong from the following chordate species, (V: vertebrata, T: tunicata and C:cephalochordata) and one echinoderm (E):

- *Anolis carolinensis* (V)
- *Branchiostoma belcheri* (C)
- *Branchiostoma floridae* (C)
- *Ciona robusta* (T)
- *Ciona savignyi* (T)
- *Danio rerio* (V)
- *Eptatretus burgeri* (V)
- *Petromyzon marinus* (V)
- *Strongylocentrotus purpuratus* (E)
- *Xenopus laevis* (V)
- *Xenopus tropicalis* (V)

We are going to test all the capability of `miRNAture`, using at the same time all the available modes: `Blast`, `HMM`, `Infernal` and the final concatenation with `Final`. Specifically for the pairwise-comparisons with `Blast` mode, we are going to use only 3 strategies: 1,9,10 and the final concatenation and comparison with `ALL`, but feel free to choose more or less strategies.

Then, just let it run typing:

```
$./run_miRNAture.sh homology
```

A long descriptive output will be printed on the screen. Keep an eye on the `Results/` folder where the action is taking place.

> **Note:** Keep in mind that `run_miRNAture.sh` was created as an example to run `miRNAture` but it does not mean that is the only way to do that. Change it according your requirements.

Homology search results

To refer directly to the results, type:

```
$ cd ../Results/
$ tree -L 1
Results/
├── Blast
├── Final_Candidates
├── HMMs
├── Infernal
├── miRNAture_log_190609072022.log
├── miRNAture_log_22505108072023.log
├── miRNAture_log_23590008072025.log
├── miRNAture_log_4209072026.log
└── mirnature_runLatch.sh
```

If everything goes well, you could see 4 log files `miRNA_log_*.log`, a script generated automatically to run the search strategy on `miRNAture` (`mirnature_runLatch.sh`) and the folders with homology comparisons: `Blast/`, `HMMs` and direct structure comparison: `Infernal` and the `Final_Candidates` with the final set of homology predicted miRNAs. Next, go directly to the `Final_Candidates` folder:

```
$ cd Final_Candidates/
$ tree -L 1
├── all_RFAM_Latch_Final.ncRNAs_homology.txt
├── all_RFAM_Latch_Final.truetable
├── all_RFAM_Latch_Final.truetable.discarded.table
├── all_RFAM_Latch_Final.truetable.joined.table
├── all_RFAM_Latch_Final.truetable.joined.table.db
├── all_RFAM_Latch_Final.truetable.temp
└── Fasta
```

Where the most important file is `all_RFAM_Latch_Final.ncRNAs_homology.txt`, which reported all the merged candidates to miRNAs on the subject contigs from coelacanth. The results are summarised on the following table:

Contig	Length (Mb)	Numb. miRNAs	miRNAture Pred.
JH126571.1	5.98145	5	122
JH126620.1	3.03251	8	106
AFYH01291077.1	0.00106	1	0

The final results could be discriminated by the annotation method (Blast, HMM or Infernal):

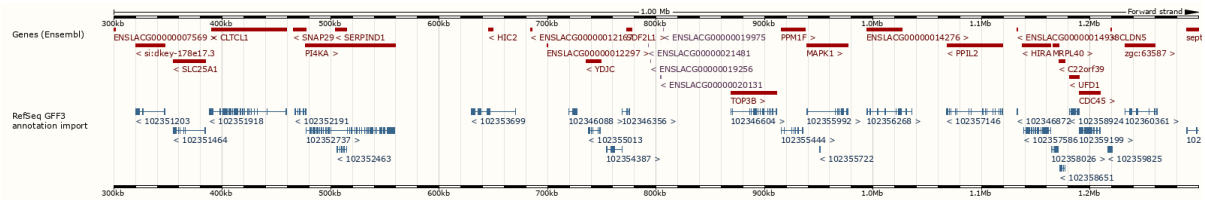
Contig	Blast	HMM	Infernal	miRNAture Pred.
JH126571.1	22	7	93	122
JH126620.1	35	6	65	106

and even, this set of computational annotations could be visualized on a broad genome context, generating for example a BED file and uploading it at the Coelacanth Ensembl Genome Browser, using some Linux commands as follows:

```
$awk '{print $1"\t"$6"\t"$7"\t"$8"\t"$2"\t"$3}'
all_RFAM_Latch_Final.ncRNAs_homology.txt > predicted_miRNAture.bed
```

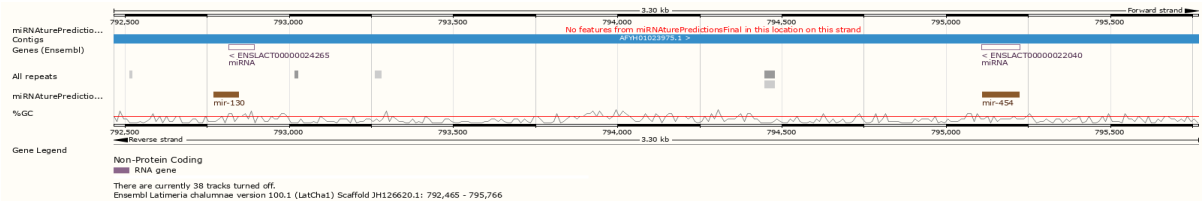
Next, just upload the track on the corresponding Genome Ensembl hub (as explained in more detail here) as a Custom Track.

Certainly, after uploading this miRNAs coordinates you would visualize this results:

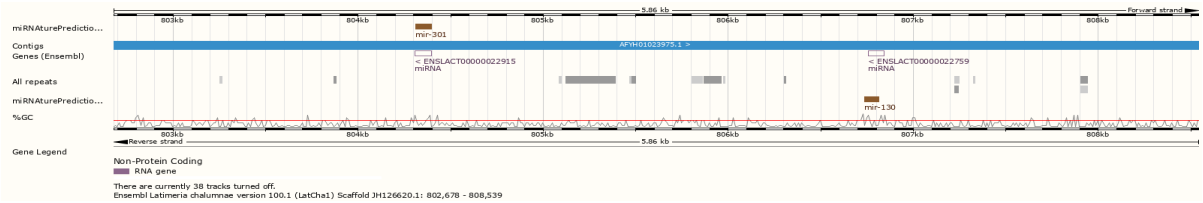


This image corresponds to the region `JH126620.1:788915-822338`, which according to the Ensembl annotation, exists 4 miRNA genes of the families: mir-130 (ENSLACG00000021481), mir-454 (ENSLACG00000019256), and two of mir-130 (ENSLACG00000020131, ENSLACG00000019975)

Here, `miRNAture` detected the same families, with overlapping regions on the previously reported miRNAs on *L. chalumnae*.



And this is the second cluster, with two families, the overlapping is the same but in one miRNA the family prediction and the strand differ:



For that reason, those candidates required a complementary evaluation of their current detection and correct positioning of the *mature* miRNA sequences. As a final result, you could check that all the reported miRNAs on the contigs JH126571.1 and JH126620.1 were identified. The reported miRNA on AFYH01291077.1, was predicted as a miRNA using RFAM, but currently there is no information about the family or mature products. `miRNAture` detected this candidate on the direct Infernal searches, but it did not show an acceptable homology (for mir-105 family) and folding values (Bitscore: 13.4 and E-value 7.8), see file `Results/miRNA_predictionInfernal/Latch/RF00670_Latch.tab`.

On the other side, `miRNAture` detected new candidates that currently are not reported on the genome annotation.

Validation of miRNA candidates

An additional output was generated on the `Final_results/` folder and contained all the resulted fasta sequences from the last 228 hairpin candidates, organized by their Rfam family. Based on those regions, validated by sequence and structure homology, the idea is to evaluate the annotation of candidate *mature* regions that are contained in this hairpin-loop and validate their annotation with an additional layer, supported by the structural alignments of sequences selected from other organisms.

To do that, please execute again the script:

```
$./run_miRNAture.sh validate
```

which essentially have the same input parameters, except for the `-stage validation` flag that was changed to tell `miRNAture` that the second stage have to be activated.

In this step, each detected miRNA candidate were grouped by their Rfam miRNA family. Based on this reference, previously calculated data from the family is retrieved. This input data, required to perform the correction of the *mature* sequences using the `MIRfix` program, was inferred as a product of this study[1] and comprises this set of files:

- The set of Rfam hairpin sequences.
- The mature sequences annotated for each Rfam hairpin sequence.
- The genomes/contigs/sequences that contained the Rfam sequences.
- A mapping file, which explicity declares the relation between hairpin and their mature sequences.

For more details refer to the `MIRfix` repository . Automatically, `miRNAture` structure all your data and generate the required input files to perform the *mature* annotation.

3.2 License

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[1] From the *seed* sequences from `Rfam` v.12.2 and additionally the sequences from `Rfam` and `miRBase` that reported *mature* sequences.

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CHAPTER

FOUR

INDICES AND TABLES

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