miRNAture

Release 1.0.0

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CONTENTS:

Computational detection of microRNA candidates

CHAPTER

ONE

INTRODUCTION

MicroRNAs (miRNAs) have been characterized as important regulators in almost all animals and plants, as well as in unicellular eukaryotes **:cite:`Moran:17`**. Since their discovery in 1993 **:cite:`Lee1993`** and subsequently their recognition as a biological entity later from 2000 **:cite:`Reinhart2000`**, microRNAs were identified as key regulators on the temporal control of heterochronic genes on the nematode *Caenorhabditis elegans* as well as in another metazoan species, too **:cite:`Pasquinelli:00`**. This recognition was 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 isolated locus or co-expressed as polycistronic miRNA transcripts **:cite:`Lagos-Quintana:2001,Lau:2001,Lee:2001,Reinhart2000`**.

Currently, 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 **:cite:`Bartel:18`**. 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 **:cite:`Zeidler:2020`**. 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 **:cite:`Bartel:18`**).

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 **:cite:`Kozomara:19`** and the corresponding mature products were estimated ~2300 **:cite:`Alles:19`**. Focusing on the *confidently canonical* miRNAs reported in **:cite:`Fromm:15`**, 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 **:cite:`Price:11`**. 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 **:cite:`Tarver:18`**. On the other hand, the inclusion of the consensus structure complements the homology search methods, for example using covariance models (CMs) **:cite:`Eddy:94, Gardner:2009`**. 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 consistently.

In this computational approach, focused on the *canonical* miRNAs processed by Drosha and Dicer, we improve on ideas from MIRfix **:cite:`Yazbeck:19a`** 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

INSTALLATION

The easiest way to install `miRNAture` is through *conda*. To do so, please first install Conda[1].

To speed up installation of dependencies and packages we suggest to use mamba[2], 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.

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

CHAPTER

THREE

QUICK START

3.1 Input files

The most important input file is a DNA sequence. This could be a multifasta sequence that belongs from a common specie (i.e. complete genome or group of particular sequences). At the same time, previous to execute **miRNAture** a you have to download a pre-calculated dataset (as indicated on *Pre-calculated datasets* Section) that contains default data as CMs, HMMs, and required files to perform mature prediction. Once located in your computer, the path might be indicated with the flag `-dataF`.

3.1.1 Activate the `mirnature` environment

```
conda activate mirnature
```

3.1.2 Run `miRNAture`

A complete mode should be run as follows:

```
./miRNAture -stage complete -dataF <Precalculated_folder> \
            -speG <Target Genome> -speN <Specie_name> \
            -speT <Tag_specie> -w <Output_dir> \
            -m <Mode> (-str <Blast_strategy>) \
            -blastq <Blast_queries_folder>
```

But it is always recommended to look up specific parameters. Do not use the default parameters for all experiments.

3.1.3 Output files

Final predicted miRNAs will be written on the `<Output_dir>` indicated with the `-w` flag. The final candidates are described on the folder `Final_miRNA_evaluation/` as follows:

```
Final_miRNA_evaluation/
├── Fasta/
├── MFE/
├── miRNA_annotation_<Tag_specie>_accepted_conf.bed
├── miRNA_annotation_<Tag_specie>_accepted_conf.gff3
├── miRNAture_summary_<Tag_specie>.txt
└── Tables/
```

Inside this folder, **miRNAture** will create 3 folders containing their correspondent results: sequences in `fasta` format (`Fasta/`), minimum free energy and lengths from described sequences (`MFE/`) and the supporting information ordered in tables for each annotated candidate (`Tables/`). Additionally, associated genomic positions for the miRNA candidates are reported in `BED` and `GFF3` formats and a summary file, `miRNAture_summary_<Tag_specie>.txt`, that describes overall descriptive statistics from found miRNA families.

3.1.4 Pre-calculated datasets

Pre-calculated data composed by miRNA CMs, HMMs and required input files to perform mature annotation has to be downloaded before run the full `miRNAture` pipeline. Available datasets are listed below:

- **NEW**: Curated metazoan families from miRBase v.22.1, available to the structural validation stage.
- Required data to re-annotate human miRNAs: include CMs and HMMs build from miRBase without human sequences. Stored in Zenodo here.

CHAPTER

FOUR

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. Merging and consolidating task from multiple search strategies is done automatically by `miRNAture`, throwing at the the end of the *Homology searches* stage, a list of regions that reported highest scores based on selected homology searches. Those candidates passed designed filters (see in more detail in Fig. 4.1), to be considered as homologs by the applied computational searches.

Further structural and microRNA-specific evaluations are covered on the *Mature evaluation* step, which makes use of an updated version of the original `MIRfix` pipeline **:cite:`Yazbeck:19a`**. At this step, `miRNAture` evaluates the identity at family level of the homology candidates found in the previous step. Based on that, makes use of the reported precursor, mature and genomic information contained on the `miRBase` database. Specially for this step, this curation step is prepared and reported with each release, allowing the user to perform the best mature positioning and assignment on their predicted precursor sequences. Please refer to the *Appendix* section to know more details about the curation process of the `miRBase` database and the generation of associated data. At this point, for each of those precursors, `MIRfix` will try to:

- Assign the best-fitting mature sequence from those reported for the discovered miRNA family.
- Predict the position of the miR* and correct the precursor sequence, based on the assigned mature.
- Evaluate on a multiple structural alignment, the fit of the new annotated precursor in regard existing annotated miRNAs classified in the same family.

Those results are feeded onto additional evaluation steps, that would assign a confidence to each precursor with its associated mature(s), namely: High, Medium or No confidence.

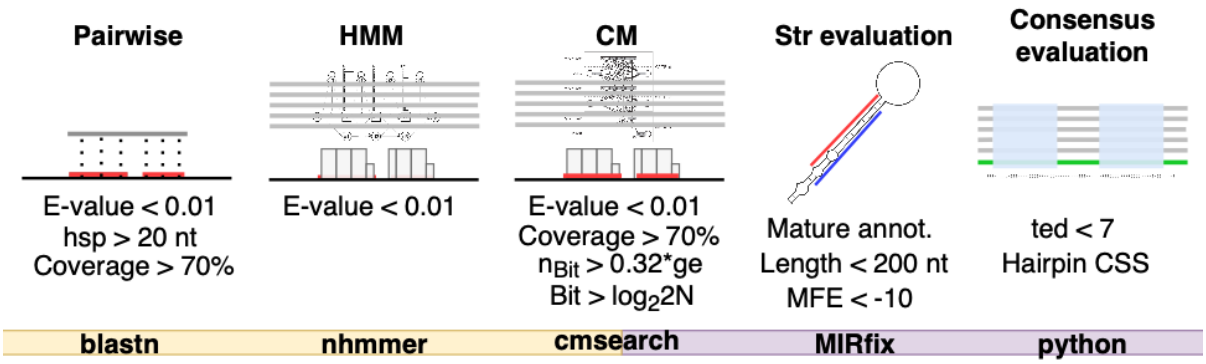

Fig. 4.1: Designed homology/strucrure filters in `miRNAture`. Specific programs used for each mode in parenthesis. Ann.: Annotation, SS: Secondary structure. CSS: Consensus secondary structure. ge: gathering cutoff from Rfam family. nBit = Bitscore/ge. ted: tree edit distance between default miRNA and modified multiple stockholm alignments. MFE: Minimum free energy. HSPs: high scoring pairs.

In the *complete* mode, `miRNAture` will report the following output files:

- `GFF3` and `BED` files of the precursors with their mature sequences.
- `Fasta` sequences from miRNA precurspors.
- A summary table describing features of found miRNAs, such as: their *loci* number, family classification and their confidence.

Current workflow is depicted in Fig. 4.2:

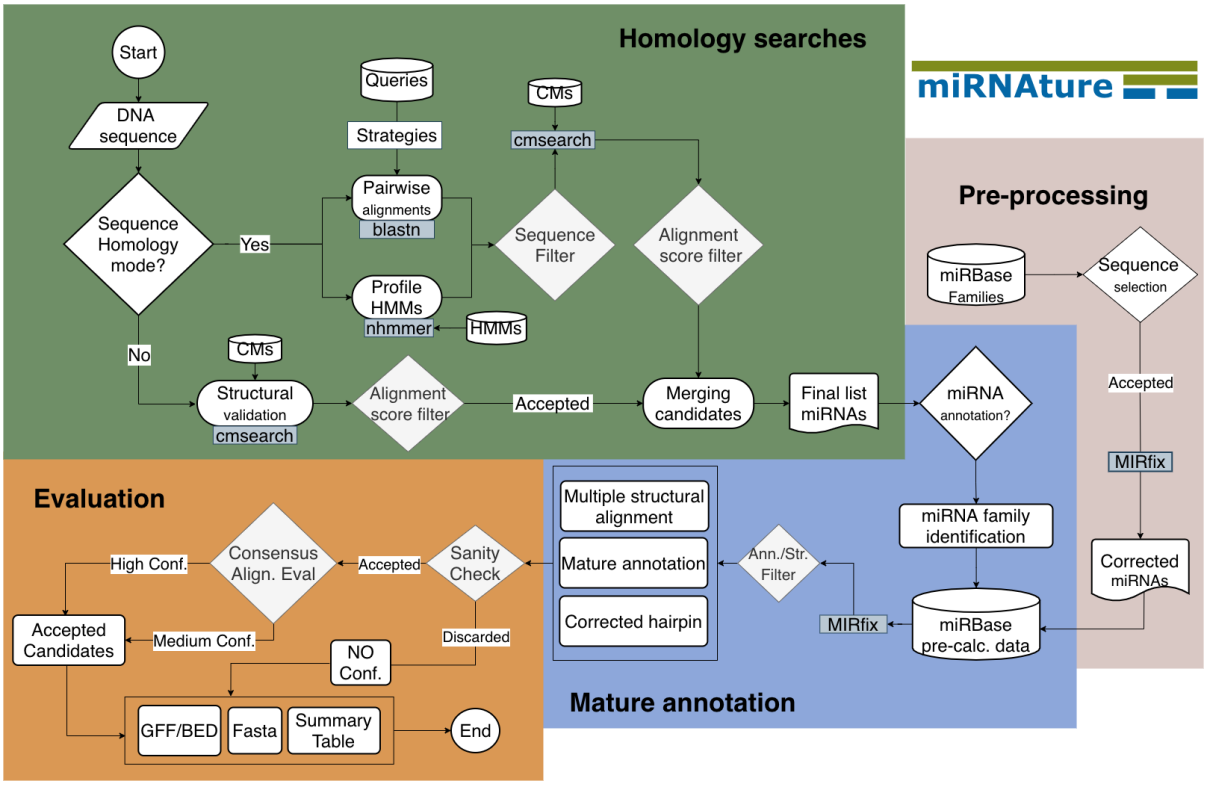

Fig. 4.2: General `miRNAture` workflow.

CHAPTER

FIVE

TUTORIAL

Through 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. All the required files to execute this tutorial are included in the `miRNAture` source files in the *miRNAture/Tutorial* folder.

5.1 Annotating let-7 on coelacanth

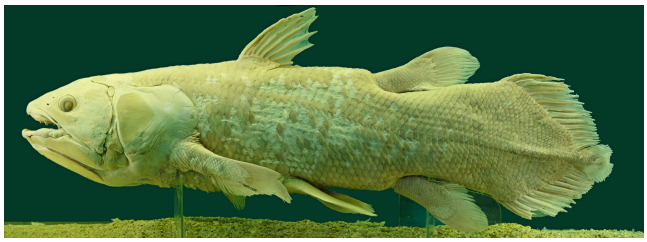

Fig. 5.1: *Latimeria chalumnae*. Source: Alberto Fernandez Fernandez / CC BY-SA

Based on the miRNA annotation retrieved from `Ensembl` release 100, the coelacanth genome featured 8 let-7 locus distributed along 6 contigs. For purposes of this tutorial, 3 contigs were selected with variable number of miRNA/let-7 annotations, as follows:

Contig	Length (Mb)	Numb. miRNAs	Let-7 loci
JH126571.1	5.981	5	1
JH129429.1	0.248	3	3
AFYH01291077.1	0.001	1	0

The main goal is the identification of the let-7 loci on the referred contigs. To do so, the *Tutorial* folder contained all the input files in *Data/*, required wrapper to *miRNAture* on *Code/* and a *Results/* folder where all prediction will be stored. As you can imagine, homology comparisons are prone to create both, a high number of input and output files. *miRNAture* avoids manual curation to the detected hits, life is too short to perform all of those steps by hand!

5.1.1 Folder structure

The folder tree on miRNAture looks like:

```
$ tree -L 1 miRNAture/
miRNAture/
├── Build.PL
├── Changes
├── ignore.txt
├── index.md
├── lib/
├── LICENSE
├── MANIFEST
├── META.json
├── META.yml
├── mirnature_logo.png
├── miRNAture-Manual/
├── miRNAture.yml
├── README
├── README.md
├── README.rst
├── script/
├── t/
├── Tutorial/
└── xt/
```

Our target folder is located in `Tutorial/`:

```
$ cd Tutorial/
$ tree -L2 .
Tutorial/
├── Code
│   ├── list_miRNAs_to_search.txt
│   ├── Precalculated-Data-tutorial
│   ├── tutorial_test_selected_models.sh
│   └── User_Test_Data
├── Data
│   ├── latimeria_chalumnae_genome.fa
│   └── 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 contigs from coelacanth in a multi-fasta file:`latimeria_chalumnae_genome.fa`. In the same folder, in `QueriesToTest/` let-7 annotations from 11 metazoans[1] were provided as queries.

Note: Together with the query files, the file *queries_description.txt* is required to control which dataset of sequences that will be used by the *blastn* comparisons. Three columns are needed to be recognized:

<Name_fasta_file.fa> miRNA <Origin_of_sequence>

The first one corresponds to the file name, the second one have to be miRNA, the third one is the name of the source specie in the format: *Genera specie*. If you do not know the source, a valid name would be: Unknown specie. If

[1] *Anolis carolinensis*, *Branchiostoma belcheri*, *Branchiostoma floridae*, *Ciona robusta*, *Ciona savignyi*, *Danio rerio*, *Eptatretus burgeri*, *Petromyzon marinus*, *Strongylocentrotus purpuratus*, *Xenopus laevis* and *Xenopus tropicalis*.

ommited, miRNAture will create automatically this file using all fasta files in this folder with an Unknown origin.

The Results/ folder will conserve all the output files generated by miRNAture.

5.1.2 Input files

To run miRNAture just go directly to Code/ folder:

```
$ cd Code/
$ tree -L 1 .
.
├── list_miRNAs_to_search.txt
├── Precalculated-Data-tutorial
├── tutorial_test_selected_models.sh
└── User_Test_Data
```

In this path, the tutorial_test_selected_models.sh file is bash script that will organize all our code to run miRNAture. This way is preferred in terms of reproducibility means of your computational experiments. This code will give you a general idea to run miRNAture, let's explain this in detail:

```
#!/bin/bash

current=$( pwd )
specie_tag="Lach"
specie_genome="$current/../Data/latimeria_chalumnae_genome.fa"
specie_name="Latimeria_chalumnae"

workdir="$current/../Results"
mkdir -p $workdir
mode="Blast,HMM,Infernal,Other_CM,Final"
strategy="5,6,ALL"
blastQueriesFolder="$current/../Data/QueriesToTest"
user_models="$current/User_Test_Data"
data_precalculated_folder="$current/Precalculated-Data-tutorial"

### Step by step: homology->validation->evaluation->summarise
# Run only homology-searches
#miRNAture -stage homology -sublist $current/list_miRNAs_to_search.txt \
#    -dataF $data_precalculated_folder -speG $specie_genome -speN $specie_name \
#    -speT $specie_tag -w $workdir -m $mode -pe 0 -str $strategy \
#    -blastq $blastQueriesFolder -rep relax,150,100 -usrM $user_models
# Run detection matures
#miRNAture -stage validation -dataF $data_precalculated_folder -speG $specie_genome \
#    -speN $specie_name -speT $specie_tag -w $workdir -m $mode -pe 0 -usrM $user_models
# Run the complete analysis
#miRNAture -stage evaluation -dataF $data_precalculated_folder -speG $specie_genome \
#    -speN $specie_name -speT $specie_tag -w $workdir -m $mode -pe 0
# Create summarise report
#miRNAture -stage summarise -dataF $data_precalculated_folder -speG $specie_genome \
#    -speN $specie_name -speT $specie_tag -w $workdir -m $mode -pe 0

# Run miRNAture complete
```

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```
miRNAture -stage complete -sublist $current/list_miRNAs_to_search.txt \
    -dataF $data_precalculated_folder -speG $specie_genome -speN $specie_name \
    -speT $specie_tag -w $workdir -m $mode -pe 0 -str $strategy \
    -blastq $blastQueriesFolder -rep relax,150,100 -usrM $user_models
```

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.

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

Declare the name of input and output locations. This will help to assign `miRNAture` flags and easily reproduce the experiment. In this case, we used the following options (flags indicated in parenthesis):

- Processing stage (`-stage`): Running stage on `miRNAture`. In this case was selected `complete` to run all the stages. To run step by step, this flag accepts: `homology`, `validation`, `evaluation` and `summarise`. You should run all of them in this order to obtain the same final results as `complete` option.
- Subset of miRNA models to run (`-sublist`): Subset of miRNA families references to be searched on the target sequence. See `list_miRNAs_to_search.txt` file as an example. If not provided all miRNA RFAM models will be searched.
- Pre-calculated data location (`-dataF`): Location of pre-calculated data required by `miRNAture`. It included hidden markov, covariance models and curated input files to annotate mature sequences[2].
- Specie genome (`-speG`): Current target sequence.
- Specie name (`-speN`): Scientific name of the specie which belongs the subject sequence(s).
- Specie tag (`-speT`): 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).
- Working directory (`-w`): Output directory, final path of `miRNAture` results.
- Running mode (`-m`): Select at least one, or any combination of the miRNA search strategies between: `Blast`, `HMM`, `Infernal` and `Other_CM`. At the same time, to merge the complete results from those homology search modes, write at the end `Final`.
- Parallel jobs using SLURM (`-pe`): Activate (1) or not (0).
- Blast strategies (`-str`): Write the numbers of desired `blastn` strategies. Possible strategies are: `1,2,3,4,5,6`. To merge all results put at the end `ALL`.
- Path of `blastn` queries (`-blastq`): Declare the path of annotated query sequences of miRNAs. In this case is enough to indicate the folder name.
- Homology repetition detection (`-rep`): Setup number of maximum loci number that will be evaluated by the mature annotation stage. By default, miRNAture will detect miRNA families that report high number of loci (> 200 loci). Then, it will select the top 100 candidates in terms of alignment scores, as candidates for the validation stage (`default,200,100`). Modify this values using `relax,Number_Loci,Candidates_to_evaluate`.
- User hidden markov/covariance models (`-usrM`): Directory with additional hidden Markov (HMMs) or covariance models (CMs) provided by the user to be searched on the target sequence.

Then, run `miRNAture` through this script:

```
$ ./tutorial_test_selected_models.sh
```

[2] Pre-calculated data should be downloaded from https://zenodo.org/record/4531376#.YDqO4-bTVTZ

Note: The list of complete flags can be found typing: `miRNAture -h` or `miRNAture -man`.

CHAPTER

SIX

APPENDIX

6.1 Pre-processing miRBase data:

The `MIRfix` pipeline **:cite:p:`Yazbeck:19a`** provides the general core of functions to curate *bona fide* metazoan microRNA annotations. To make use of this curation process, it is fundamental to organize the input data in a specific format, as referenced in more detail in **:cite:`Yazbeck:19a`**. In summary, it is required:

- A set of precursor sequences with their associated mature sequences.
- Genome sequences from which miRNAs were annotated.
- A relation file that describes the relation between precursors and their annotated matures.

Additional parameters are required, but they did not depend from external information/databases.

On `miRNAture` the source of the curation data has been obtained from a re-evaluation of the annotations deposited on `miRBase` v.22.1 **:cite:`Kozomara:19`**. In this version, `miRBase` accounted for a set of X *canonical* and *non-canonical* miRNA families, from which Y are constituted by metazoan sequences. Internally, `miRNAture` performs an evaluation of the *canonical* model, that relies on the correct positioning cleavages performed by Drosha and Dicer at the precursor maturation steps. Computationally, this is translated on a correct position of the mature sequence, accurate delimitation of precursor, and a phylogenetic support, addressed by the construction of family *mature-anchored* structural alignments. As previously reported for the `Rfam` miRNA families **:cite:`VelandiaDiss:2022`**, an iterative assessment involves a selection of sequences, consistent criteria to evaluate the miRNAs and their mature products, and generation of probabilistic models derived from anchored-alignments to search additional candidates that would incorporate defined curation criteria. This criteria was inherited to perform an evaluation of the `miRBase` metazoan families and generate the corrected dataset that `miRNAture` uses to evaluate new candidates and their maturation entities.

As a toy example, the family miR-17 (MIPF0000001) was selected to demonstrate the assessment steps performed over all pre-calculated dataset used by *miRNAture*. As reported in `miRBase` this family is composed by 239 miRNA precursors derived from 39 vertebrate species. Through the filtering approach the following subsetting steps are considered:

- Remove non-metazoan sequences.
- Filter duplicates (which share 100% identity) and select one representant sequence.

In this family, 117 duplicated sequences where recognized. For instance the sequence bta-mir-18a (MI0004740) from *Bos taurus* has shown 21 orthologs, as follows:

And some corresponding alignments:

Remaining 122 families were subject of a structural assessment by `MIRfix`, which filtered 4 sequences based on the incorrect miRNA folding in regard their annotated mature sequences, and one sequence contained a bad positioned mature sequence in the reported precursor, a successful extension of the precursor based on the miR and miR* prediction, rescued the candidate.

Accession	ID	Query start	Query end	Subject start	Subject end	Strand	Score	Evalue
MI0000072	hsa-mir-18a	1	71	1	71	+	355	3e-23
MI0002455	ssc-mir-18a	1	71	9	79	+	355	3e-23
MI0002966	ggo-mir-18a	1	71	1	71	+	355	3e-23
MI0002972	lca-mir-18	1	71	1	71	+	355	3e-23
MI0002978	age-mir-18	1	71	1	71	+	355	3e-23
MI0002984	ppa-mir-18	1	71	1	71	+	355	3e-23
MI0002990	ppy-mir-18a	1	71	1	71	+	355	3e-23
MI0002996	ptr-mir-18a	1	71	1	71	+	355	3e-23
MI0003002	mml-mir-18a	1	71	1	71	+	355	3e-23
MI0003008	sla-mir-18	1	71	1	71	+	355	3e-23
MI0003014	lla-mir-18	1	71	1	71	+	355	3e-23
MI0003020	mne-mir-18	1	71	1	71	+	355	3e-23
MI0004740	bta-mir-18a	1	71	1	71	+	355	3e-23
MI0005355	mdo-mir-18a	1	71	2	72	+	355	3e-23
MI0010324	cfa-mir-18a	1	71	9	79	+	355	3e-23
MI0018783	aca-mir-18a	1	71	14	84	+	355	3e-23
MI0028682	efu-mir-18	1	71	6	76	+	355	3e-23
MI0029402	cpi-mir-18a	1	71	8	78	+	355	3e-23
MI0029673	ami-mir-18	1	71	7	77	+	355	3e-23
MI0030162	pbv-mir-18a	1	71	13	83	+	355	3e-23
MI0030660	chi-mir-18a	1	71	7	77	+	355	3e-23
MI0031381	oha-mir-18a	1	71	5	75	+	346	2e-22

Fig. 6.1: Identified orthologs from bta-mir-18a on vertebrates.

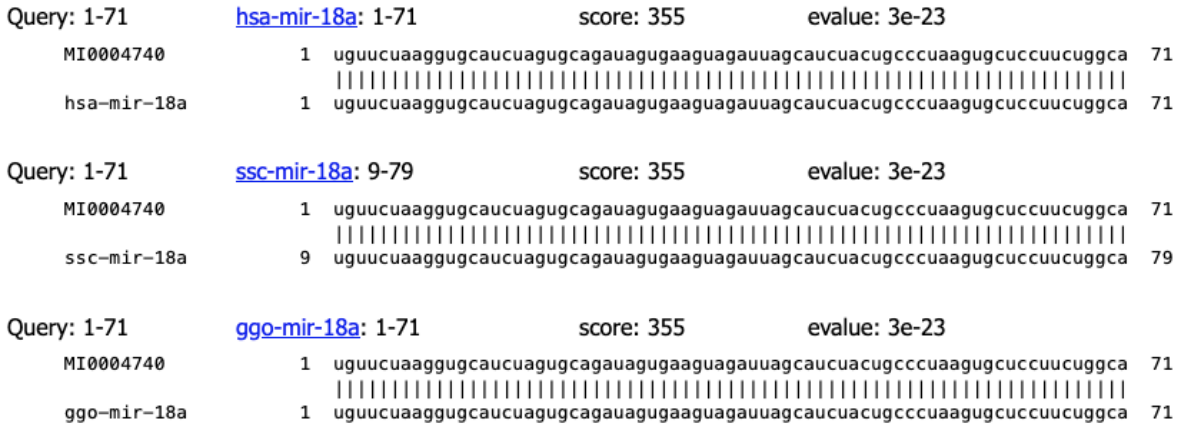

Fig. 6.2: Alignments as evidence of 100% identity.

Category	Accession numbers
Bad position mature sequences	MI0004822
Filtered sequences	MI0012797, MI0012947, MI0019542, and MI0013837

At the end of the assessment 118 sequences passed all filters to be considered into the curation dataset used on `miRNAture`.

The same approach curated all metazoan miRNA families from `miRBase` (1415), validating about 79% (1111) of the families and setting the curation dataset used on `miRNAture`.

6.2 Construction of Hidden Markov and Covariance Models:

As described in **:cite:`Velandia:2021`**, a set of quality-filtering steps could be used to construct family structural alignments and their corresponding covariance models (CMs). In this case, to build new structural alignments from `miRBase` sequences, we selected all sequences from metazoan species and removed all of those from studied organisms. Given that curated subset, a genetic algorithm was used to maximize the quality the final structural alignment. To do so, filtering miRNA sequences was done in function of: Identity percentage (I), phylogenetic distribution of sequences (C) and quality (Q)[1], where: $I = (60, 70, 80, 90, 100)$, $C =$ (Metazoa, Vertebrata, Mammalia, Primates) and $Q =$ (normal, high). An individual A_n was defined as a vector $\overrightarrow{A_n} = \begin{pmatrix} I \\ C \\ Q \end{pmatrix}$, which return a structural alignment using `MIRfix`, using selected sequences. The *fitness* function (F) to be maximized was defined through empirical observation over features inferred from generated structural alignment, as follows:

$$F = (N_{seq} + (N_{spe} * (-F_{energy})) + (N_{parts} * 10))$$

Where N_{seq} is the final number of sequences, N_{spe} is the number of species, F_{energy} corresponds to folding energy calculated using `RNAalifold` **:cite:`Lorenz2011`** and N_{parts} accounts the number of additional (> 1) stem-loops on the reported consensus structure. The initial population was $A_p = 40$, used operators were: *Selection* = Tournament, $n = 39$; *Crossover* = Single point, probability=0.7; *Mutation* = Displacement mutation, probability=0.1. The implementation were performed in `Python` v3.7.9 using `deap` package **:cite:`Fortin:2012`**.

Finally, hidden Markov (HMMs) and covariance (CMs) models were build as described in **:cite:`Velandia:2021`** using `RNAalifold` **:cite:`Lorenz2011`** and `Infernal` package v.1.1.2 :cite:``.

[1] Confidence of the annotation assigned by `miRBase`, see *https://www.mirbase.org/blog/2014/03/high-confidence-micrornas/*

CHAPTER

SEVEN

LICENSE

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Preamble

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CHAPTER

EIGHT

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