miRNAgFree: prediction and profiling of novel microRNAs without genome assembly

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Abstract

The prediction of novel miRNA genes generally requires the availability of genome sequences in order to assess important properties such as the characteristic hairpin-shaped secondary structure. However, although the sequencing costs have decreased over the last years, still many important species lack an assembled genome of certain quality. We implemented an algorithm which for the first time exploits characteristic biogenesis features like the 5' homogeneity that can be assessed without genome sequences. We used a phylogenetically broad spectrum of well annotated animal genomes for benchmarking. We found that between 90-100% of the most expressed miRNA candidates (top quartile) corresponded to known miRNA sequences.

Keywords: Bioinformatics, reference genome, prediction, de novo, genome

Availability: http://bioinfo2.ugr.es:8080/ceUGR/mirnagfree/

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1 Introduction

MicroRNAs (miRNAs) have important roles in many biological processes (Bushati and Cohen, 2007) and they possess a huge potential to become prominent biomarkers as they can be detected

in nearly every bodily fluid (Cortez et al., 2011). The miRNA expression profiling can be routinely carried out by means of micro-arrays or next-generation-sequencing if the mature and pre-miRNA sequences are available. Therefore the determination of the miRNA reference sequences is an essential task. Generally, genome sequences are required and many different programs are available for the prediction of novel miRNAs like miRanalyzer and miRDeep2 (Hackenberg et al., 2011; Friedländer et al., 2012) or miRCandRef which works with unassembled reads (Fromm et al., 2013). There are also some programs available for the prediction without genome like miRMiner (Wheeler et al., 2009) which is based on homology and, miRPlex (Mapleson et al., 2013) and miReader (Jha et al., 2013) based on machine learning using different duplex features.

Here we present a novel miRNA prediction approach based on biogenesis features, such as the known 5' homogeneity, and duplex features like mean free energy which do not require a genome assembly to be assessed. We found that, in general, biogenesis related parameters are far more discriminative than duplex related structural parameters. We observed that a high percentage of the top expressed miRNA candidates in animals correctly match actual guide sequences while the prediction without genome in plants seems to be more complex, leading to much lower specificities. Our approach outperforms previous similar attempts because microRNA biogenesis features were taken into account. We benchmarked miRNAgFree using a set of species with high quality genomes (including *H.sapiens, mus musculus, C.elegans, D.Rerio)* using publicly available datasets. When measuring the specificity on the guide strand of the microRNA we obtained over 90% accuracy for the most expressed quartile of duplexes.

2 Main features and implementation

miRNAgFree is a piece of software that allows for mature microRNA prediction using sRNAseq/miRNAseq/sncRNAseq without needing a genome or miRNA sequence libraries. This tool is therefore ideal for non-model species with genomes yet to be sequenced or for those lacking an appropriate quality. The software uses the sRNAbench preprocessing and therefore accepts several input files:

- Adapter trimming can be performed and miRNAgFree accepts *fastq*, *fastq.gz*, read count and *fasta* input format
- A preprocessing filtering step can be included. This is useful in a number of scenarios: for example to remove unwanted ribosomal sequences or if there are some already described microRNAs that should be eliminated from the analysis. Reads mapping to the provided libraries will not be considered for downstream analysis.
- Lax parameter settings (more sensitive) and strict settings (more specific) are provided.

2.1 Implementation steps

The general workflow consists in i) preprocessing of the input reads, ii) read filtering (optional if the user provides a filter library like ribosomal RNA), iii) clustering of the reads, iv) calculation of

duplexes with RNAcofold , v) detection of microRNA-like duplexes and vi) output layer including visualization of the detected miRNA duplexes.

2.1.1 Cluster method

The mature microRNAs are normally represented at the read level by the canonical sequence (i.e. the one in miRBase) and its isomiR sequences. Therefore, in order not to predict a microRNA several times due to duplexes formed by its isomiR sequences, we first cluster together all reads. Briefly the algorithm performs the following steps on a sorted read list (descending order)

(1) Open a cluster with the most abundant read as dominant read

(2) Take the most abundant read as reference and align all other reads against it. The reads can align with a 3 nt overhang at the 5' end allowing by default 1 mismatch (suppl. figure 1a). The last nucleotides are ignored as those might be NTAs (non-templated additions)

(3) Remove all assigned reads so they are not considered in other clusters

(4) Repeat steps 1-3 until no reads are left.

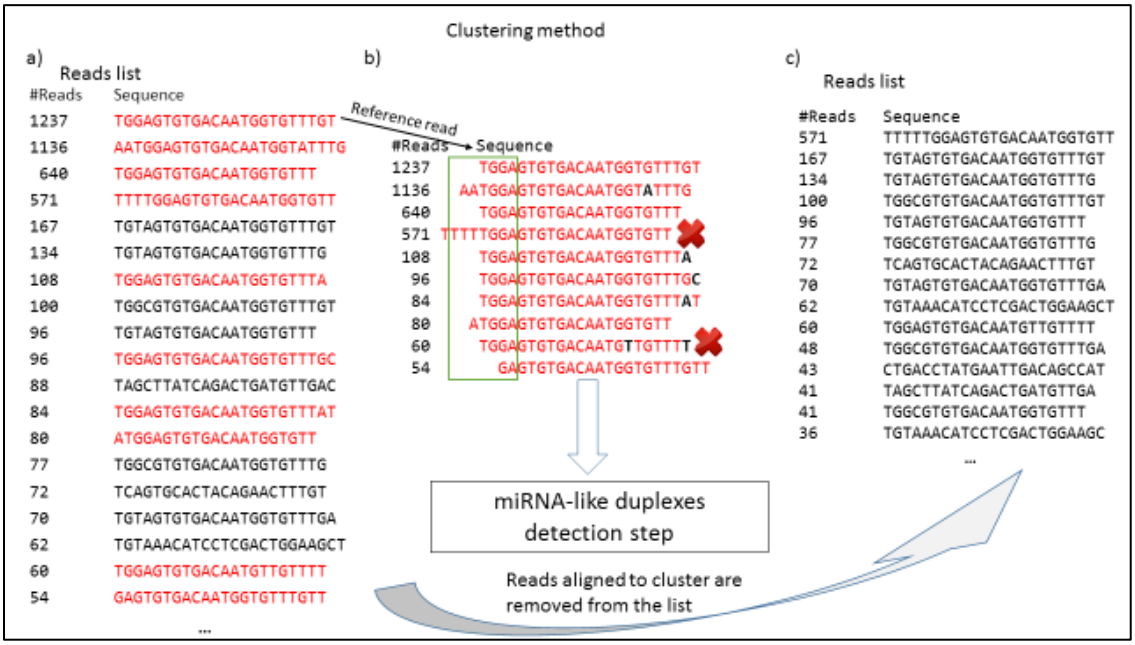


Fig 1. Iterative clustering method. a) The most expressed read opens the cluster and all reads are aligned to this sequence. b) All reads that map to the reference are part of the cluster. Reads need to start within a 3nt window around the 5' end of the dominant read (green rectangle) and 1 mismatch (nucleotides in black) is allowed by default (user parameter). c) All assigned reads are removed from the input list and the iterative steps start again at point a).

2.1.2 Detection of miRNA duplexes

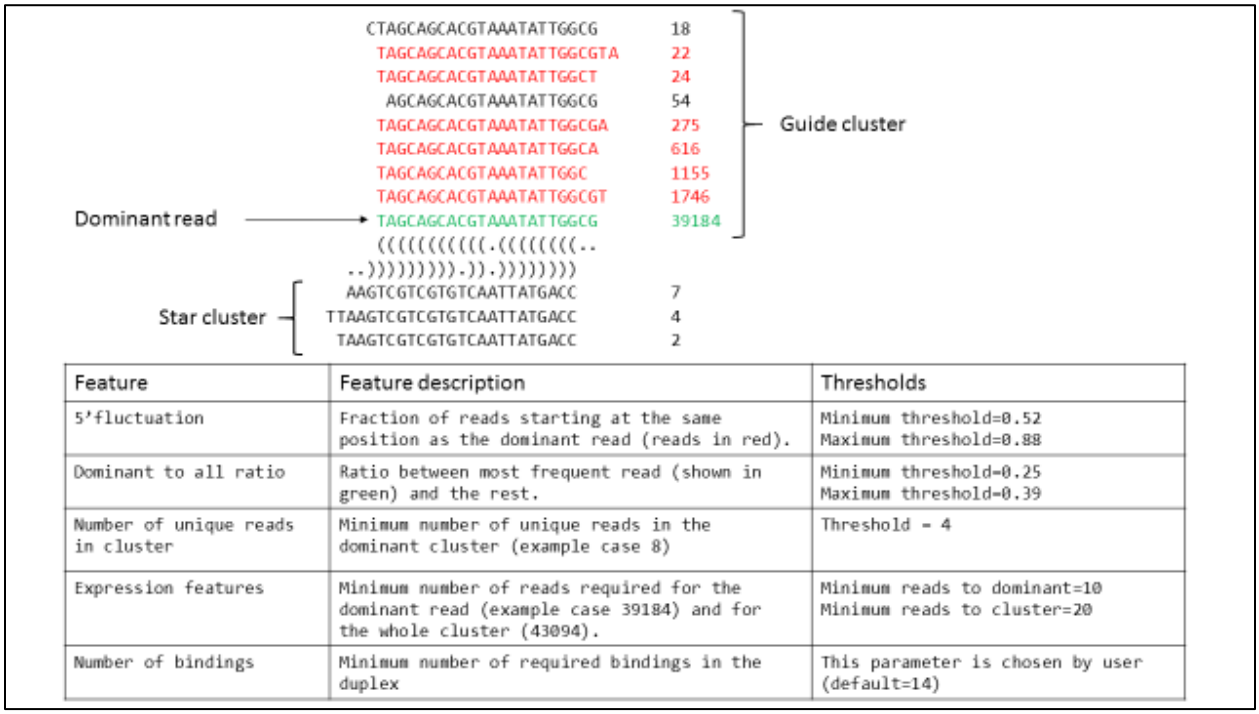
The detection of novel microRNAs consists of two steps: i) selection of clusters that resemble those formed by real guide microRNAs and their isomiRs and ii) the assignment of the most likely passenger sequence.

(1) Sort clusters by total expression value in descending order.

(2) Pick the most expressed cluster and remove it from the list and evaluate its microRNA potential based on several criteria like the 5' homogeneity or the ratio of the most expressed read to the read count sum of the cluster. The same thresholds as in (Barturen et al., 2014) were used. The user can choose between lax and strict settings for both animals and plants.

(3) For all putative cluster pairs, calculate all duplexes for the (M) most expressed reads in cluster (i) vs the (N) most expressed clusters of cluster (j) and remove all that do not have perfect 2 nucleotide 3' Drosha/Dicer overhangs (strict) or at least 1-3 nt overhang (lax mode)

(4) Sort the duplexes by energy ratio (mean free energy divided by the sequence length) and assign the energetically most favorable read and its cluster to the guide cluster.



Feature	Feature description	Thresholds
5'fluctuation	Fraction of reads starting at the same position as the dominant read (reads in red).	Minimum threshold=0.52 Maximum threshold=0.88
Dominant to all ratio	Ratio between most frequent read (shown in green) and the rest.	Minimum threshold=0.25 Maximum threshold=0.39
Number of unique reads in cluster	Minimum number of unique reads in the dominant cluster (example case 8)	Threshold = 4
Expression features	Minimum number of reads required for the dominant read (example case 39184) and for the whole cluster (43094).	Minimum reads to dominant=10 Minimum reads to cluster=20
Number of bindings	Minimum number of required bindings in the duplex	This parameter is chosen by user (default=14)

Fig. 2 Parameters and thresholds used in the miRNA-like selection

3 Results

To show the usefulness of our approach we used two strategies: i) assess the number of correct predictions by means of well annotated genomes and ii) show that species with an

unfinished genome assembly (*Fasciola hepatica*) very likely have incomplete miRNA complements. We found that the percentage of correct guide sequences ranges from 51% (C. elegans) to 91% (D. melanogaster) in animals but are generally lower in plants. The fraction of correct guide sequences increases with the threshold number of bindings, whereas the total number of predictions drops strongly, and hence the sensitivity. The prediction quality seems to be notably affected by the quality of the sequencing data. For the M. musculus data we observed a specificity increase from 50.8% to 85.2% when filtering out all reads that have a single position with lower phred score than 20. Even though we prioritized specificity to increase confidence in the yielded prediction, we still found that sensitivity ranged between 51% (most strict set of parameters) and 81% for the least strict settings (see suppl. Table 1).

Further, we found that prediction quality increases with expression value, i.e. the top 14 guide sequences in mouse and the top 44 in zebrafish are all correct (see column c in Table 1). Finally, using publically available data for *F. hepatica* (SRR1825354) we detected a member of the let-7 family which was not reported in the most recent complement using the genome assembly (Fromm et al., 2017) either due to assembly quality or structural properties which prevented its prediction (see supplementary figure 1).

Table 1. Benchmark of miRNAgFree using well annotated species.

Species	b	N	p (%)	p25 (%)	c	SRA accession
Homo sapiens	16	79	82.3	95.7	16	SRR1563015
Homo sapiens	18	24	91.7	100	22	SRR1563015
Mus musculus	16	61	85.2	93.3	14	SRR1734811
*Mus musculus**	16	118	50.8	90	14	SRR1734811
Danio rerio	16	170	75.9	100	44	SRR3953259
D. melanogaster	16	33	90.9	100	29	SRR1287661
C. elegans	16	183	51.4	91.3	40	ERR562747
A. thaliana	16	63	30.1	37.5	2	SRR5031522

b is the applied duplex bindings threshold, N the total number of predicted miRNAs, p is the proportion of correct guide sequences (annotated in miRBase 21), p25 is the same as p but just considering the top quartile of predictions, c is the number of consecutive correct predictions among the top expressed ones. * indicates that no quality filter was applied to the raw fastq file.

In summary, we tested different duplex related structural features as used in prior approaches, but we found that biogenesis features and specially the 5' homogeneity outperform those clearly. Given that the top expressed guide miRNAs are generally correct, miRNAgFree can be an important tool for miRNA research in non-model species.

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Supplementary material

Supplementary table 1. Specificity and sensitivity values obtained for the *Homo sapiens* dataset

Mode used	Maximum length allowed for candidate microRNA	Number of mismatches allowed in the clustering step	Sensitivity	Specificity
Strict mode (same as Table 1)	23	1	0.5112359551	0.79
Lax mode (keeping the rest as in Table 1)	23	1	0.6853932584	0.6338582677
Lax mode	24	1	0.7430167598	0.5906040268
Lax mode	24	0	0.808988764	0.7879464286

Suppl. Fig 1. A real example from let-7 in *F. hepatica* predicted using the publically available dataset *SRR1825354*.

```
      AGAGGTAGTGACTCATATGACTTTT 10
      CAGAGGTAGTGACTCATATGACT   11
        AGGTAGTGACTCATATGACT  18
        GAGGTAGTGACTCATATGACT 24
      AGAGGTAGTGACTCATATGACC    30
      AGAGGTAGTGACTCATATGACTTT  40
      AGAGGTAGTGACTCATATGACA    73
      AGAGGTAGTGACTCATATGACG    77
        AGAGGTAGTGACTCATATGAC 79
        AGAGGTAGTGACTCATATGA  93
     AGAGGTAGTGACTCATATGACTT   111
    AGAGGTAGTGACTCATATGACT    13386
         ((((((((((..((.((((...
         ..))))))))))..)).))))
        TGTCTCCGTCATCTAGCATAC 5
      TTGTCTCCGTCATCTAGCATAC    2
      TTAGTCTCCGTCATCTAGCATA    2
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