

Distinguishing between MicroRNA Targets from Diverse Species using Sequence Motifs and K -mers

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Abstract: A disease phenotype is often due to dysregulation of gene expression. Post-translational regulation of protein abundance by microRNAs (miRNAs) is, therefore, of high importance in, for example, cancer studies. MicroRNAs provide a complementary sequence to their target messenger RNA (mRNA) as part of a complex molecular machinery. Known miRNAs and targets are listed in miRTarBase for a variety of organisms. The experimental detection of such pairs is convoluted and, therefore, their computational detection is desired which is complicated by missing negative data. For machine learning, many features for parameterization of the miRNA targets are available and k -mers and sequence motifs have previously been used. Unrelated organisms like intracellular pathogens and their hosts may communicate via miRNAs and, therefore, we investigated whether miRNA targets from one species can be differentiated from miRNA targets of another. To achieve this end, we employed target information of one species as positive and the other as negative training and testing data. Models of species with higher evolutionary distance generally achieved better results of up to 97% average accuracy (mouse versus *Caenorhabditis elegans*) while more closely related species did not lead to successful models (human versus mouse; 60%). In the future, when more targeting data becomes available, models can be established which will be able to more precisely determine miRNA targets in host-pathogen systems using this approach.

1 INTRODUCTION

Proteins have a large influence on the phenotype and, therefore, their abundance can be fine-tuned on several levels while their dysregulation may often lead to disease. The most direct regulators of protein abundance are microRNAs (miRNAs) which are involved in post-transcriptional gene regulation (Erson-Bensan, 2014). They modulate protein abundance via interacting with messenger RNA (mRNA) thereby fine-tuning translation rates (Saçar and Allmer, 2013). To achieve this, a short stretch of nucleotides (mature miRNA; ~20 nt) serves as a recognition sequence within the RNA induced silencing complex (RISC). Post-transcriptional regulation via miRNAs is found in a wide range of species ranging from viruses (Grey, 2015) to plants (Yousef et al., 2015). Experimentally determined mature miRNAs and pre-miRNAs (their sources) are

stored in miRBase (Griffiths-Jones, 2010) and its release 21 contains about 28,000 mature miRNAs (~2,600 for human), but it has been estimated that more miRNAs may exist (Londin et al., 2015).

Unfortunately, the experimental detection of miRNAs is difficult since they can only be analyzed when co-expressed with their target mRNAs which is impossible to achieve for all miRNA-mRNA pairs at the moment (Saçar and Allmer, 2013). Therefore, computational prediction of pre-miRNAs is employed and most approaches are based on machine learning using two-class classification (Allmer, 2014; M. Saçar and Allmer, 2014). Such *ab initio* models have been established for metazoan (Allmer and Yousef, 2012) and we have shown that similar models can be trained for plants (Yousef et al., 2015). Machine learning for pre-miRNAs depends on parameterization of the biological structure and many features are available (Saçar and Allmer, 2013). We

have recently also added sequence motifs as additional features for describing pre-miRNAs (Yousef et al., 2016a). Parameterization is important to train classifiers which, based on a feature-value set, can learn to differentiate between the positive (miRNA) and the negative class. Many machine learning approaches like support vector machines (Ding, Zhou, & Guan, 2010) and random forest (Jiang et al., 2007) have been used but in general two-class classifications suffers from the lack of bona fide negative pre-miRNA examples (Khalifa et al., 2016). The same is true, if not worse, for negative examples of the targets of miRNAs. Such targets are short stretches of nucleotides complementary to the mature sequences incorporated into RISC. Experimentally supported, so called miRNA-mRNA duplexes, are available in miRTarBase (Hsu et al., 2011) and TarBase (Sethupathy et al., 2006), but there is no dataset for which it is clear that it does not contain target sites for even selected miRNAs. This is especially complicating the computational prediction of miRNA targets (Hamzeiy et al., 2014). Therefore, one-class classification has been used for miRNA target prediction (Yousef et al., 2016b).

Here we employ two class classification, but avoid the problem of missing negative data since instead of trying to determine miRNA targets we investigate the difference among miRNA targets among species. Thus, it is our aim to differentiate between miRNA targets of one species by using another species as negative training data employing only sequence-based features, which means that positive and negative classes derived from known miRNA targets. Our approach is further supported by the finding that miRNA targets are not highly conserved within vertebrate, fly, and nematode 3'UTRs (Chen and Rajewsky, 2006). For family classification of pre-miRNAs Ding et al. used n-grams (Ding et al., 2011) which is somewhat related to the problem investigated here. Ding et al. aimed to assign a miRNA to a family of miRNAs while we are determining to which species a miRNA target belongs. We further aimed to establish the evolutionary distance which allows differentiation between targets of different species. We observed a slight trend to better differentiation for species that are further apart evolutionarily, but especially mouse and rat examples present unexpected outliers which may be due to low quality data and low relative amount of data available for rat. Facilitating the differentiation of miRNA targets among species may in the future allow the investigation of communication between host and parasite (Saçar et al., 2014; Saçar Demirci et al., 2016).

2 MATERIALS & METHODS

2.1 Datasets

We downloaded all microRNAs' targets for all species available on miRTarbase with about 500 targets or more. Data for *Homo sapiens* (has), *Caenorhabditis elegans* (Cel), *Mus musculus* (Mmu), *Rattus norvegicus* (Rno), and *Bos taurus* (Bta) were downloaded from miRTarBase (Release 6.0: Sept. 15, 2015); for details see Table 1.

The miRNA-mRNA duplexes, representing miRNA targets were filtered according to sequence similarity using USEARCH (Edgar, 2010) on the sequences of each species and also on a per species basis to ensure that there is no bias due to multiple identical target sequences. We only found 74 similar sequences between Hsa and Mmu, which were removed.

Table 1: List of the species whose known miRNA mRNA duplexes were used in this study and their amounts available on miRTarBase.

| Species | Number of target sites | After Cleaning | Average Duplex Length |
|---------|------------------------|----------------|-----------------------|
| Cel | 4,029 | 2,233 | 23.2 |
| Mmu | 54,951 | 9,278 | 29.0 |
| Hsa | 317,542 | 89,752 | 25.2 |
| Rno | 658 | 532 | 22.5 |
| Bta | 489 | 393 | 24.1 |

2.2 Parameterization of MicroRNA Targets

2.2.1 K-mers

These are short stretches of nucleotides of length k which are also termed n-grams or words. Such sequence-based features were used for *ab initio* pre-miRNA detection, and may also be useful for target prediction (Yousef et al., 2016b). Formally, a k -mer is one element of the relevant alphabet, here $\{A, U, C, G\}$. A 2-mer can generate 16 different elements: AA, AC, ..., UU. Higher k have also been used (Çakır and Allmer, 2010), but here we limited k to $1 \leq k \leq 3$ leading to 84 features. As features k -mer frequencies were calculated from the target sequences divided by the k -mers in the sequence given by $\text{len}(\text{sequence}) - k + 1$.

2.2.2 Motif Features Describing MicroRNA Targets

Instead of describing exact sub sequences, motifs

allow for approximate matches including some degree of error tolerance. The MEME (Multiple Expectation Maximization for Motif Elicitation) Suite (Bailey, T. L. et al., 2009) was used to establish motifs which are short stretches of nucleotides that occur more frequently than expected by chance within the given set of sequences. MEME is based on (Bailey and Elkan, 1994) which repeatedly searches for ungapped sequence motifs within the input sequences which explains its long runtime. MEME provides regular expressions and sequence profiles to represent the motifs. Profiles are more informative than regular expressions, which is why, different from our previous works (Yousef et al., 2016a, 2015), we decided to use profiles for feature creation. For each species we discovered 100 motifs serving either as positive or negative data thus 200 motifs were available for each experiment in addition to 84 k -mers. To calculate feature scores, profiles were aligned with the target sequence and shifted along until the end of the profile reached the end of the sequence or vice versa in case the profile was longer than the sequence. At each position, a score was calculated by adding up the frequencies in the profile for matching nucleotides at their respective positions.

The motif position leading to the highest score is reported as the final score for that input sequence.

2.2.3 Feature Vector and Feature Selection

For each experiment 284 features were available, but, not all features are equally effective to train a machine learning classifier and therefore, we used KNIME (Berthold et al., 2008) to calculate information gain (Yang and Pedersen, 1997) on a per experiment basis and accepted the 100 features with highest information gain. This feature set was used during model establishment to select from the possible features in this study: A ... U ($k=1$), AA ... UU ($k=2$), AAA ... UUU ($k=3$), Motif₁, Motif₂, Motif₃, ..., Motif_n; where $n=200$.

2.3 Classification Approach

Random Forest (RF) was used for classification in this study since it outperformed support vector machines (Vapnik, 1995), decision trees (DT), and Naive Bayes (NB) in tests preceding the study. The classification approach was setup using the data analytics platform KNIME (Berthold et al., 2008).

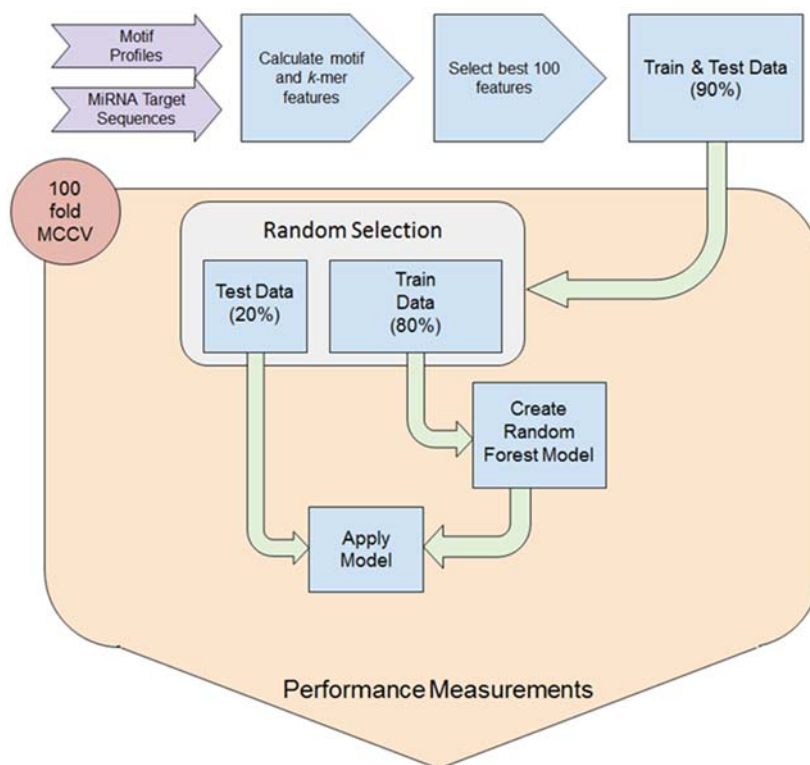


Figure 1: Workflow for model establishment. Data was transformed into a feature vector and the best 100 were selected. During the 100-fold MCCV training and testing scheme 80% randomly selected examples were used to train the classifier and 20% were used for testing. All performance measures for testing and holdout data were collected during CV and reported at the end of the workflow.

Models were trained and tested using 100 fold Monte Carlo Cross Validation (Xu and Liang, 2001) and in each fold of the cross validation (CV) the data were split into 80% training and 20% testing. During random selection, negative and positive examples were sampled in equal amounts since we showed that this approach is beneficial for model establishment in pre-miRNA detection (Sakar and Allmer, 2013). For each of the 100-fold Monte Carlo cross validation (MCCV) the performance was recorded (Figure 1).

Table 2: Number of motifs and k -mers among the top 100 features during training and testing according to the training scheme in Figure 1.

| | # of motifs | # of k -mers |
|------------|-------------|----------------|
| Mmu vs Cel | 23 | 77 |
| Rno vs Cel | 24 | 76 |
| Hsa vs Cel | 22 | 78 |
| Rno vs Hsa | 24 | 76 |
| Bta vs mmu | 26 | 74 |
| Bta vs Hsa | 26 | 74 |
| Bta vs Cel | 71 | 29 |
| Rno vs Cel | 65 | 35 |
| Rno vs Bta | 33 | 67 |
| Hsa vs Mmu | 63 | 37 |

2.3.1 Model Performance Evaluation

For each established model we calculated a number of performance measures for the evaluation of the classifier such as sensitivity, specificity and accuracy according to the following formulations (with TP: true positive, FP: false positive, TN: true negative, and FN referring to false negative classifications):

$$\text{Sensitivity} = \text{TP} / (\text{TP} + \text{FN}); (\text{SE}, \text{Recall})$$

$$\text{Specificity} = \text{TN} / (\text{TN} + \text{FP}); (\text{SP})$$

$$\text{Precision} = \text{TP} / (\text{TP} + \text{FP})$$

$$\text{F-Measure} = 2 (\text{precision} * \text{recall}) / (\text{precision} + \text{recall})$$

$$\text{Accuracy} = (\text{TP} + \text{TN}) / (\text{TP} + \text{TN} + \text{FP} + \text{FN}); (\text{ACC})$$

$$\text{MCC} = \frac{(\text{TP} * \text{TN} - \text{FP} * \text{FN})}{\sqrt{(\text{TP} + \text{FP})(\text{TP} + \text{FN})(\text{TN} + \text{FN})(\text{TN} + \text{FP})}}; \text{Matthews}$$

Correlation Coefficient (Matthews, 1975).

All reported performance measures refer to the average of 100-fold Monte Carlo Cross Validation (MCCV).

3 RESULTS AND DISCUSSION

The random forest classifier was used to establish machine learned models using an 80/20 split from random sampled and stratified training and testing

data during 100-fold MCCV (Figure 1). During feature selection generally few motifs (22-33%) were selected, but for Bta vs Cel, Rno vs Cel, and Has vs Mmu 63-71% were selected (Table 2).

In general, about 25% of the informative features were motifs which, given the low amount of examples available for some species (Table 2), was to be expected. The number of features that should optimally be used for classification was tested (Figure 2). For many tests even low number of features lead to relatively good results. To select the most suitable number of features we used species combinations which lead to slightly above 70% average accuracy since lower and higher accuracies may be biased. Therefore, we selected 100 features since for Bta vs Cel and for Rno vs Cel this number of features led to the best average accuracy (Figure 2).

The feature sets consisting of 100 parameters were then used to establish models to differentiate between miRNA targets from one versus the other species (Table 3).

Table 3 indicates that distantly related species (Figure 3) are easier to differentiate using the trained models. Examples are Mmu vs Cel, Hsa vs Cel, Bta vs Cel, and Rno vs Cel. However, Rno vs Mmu which are the perhaps most closely related species (Figure 3) in this study achieved an unexpectedly high accuracy whereas Hsa vs Mmu and Rno vs Bta were according to expectations. Table 3 provides the average accuracy and other model performance measures. To confirm that the 100 fold model training and testing is of low variance, accuracy was recorded at each step (Figure 4). The distribution was best for Mmu vs. Cel and worst for Rno vs. Bta judged by the interquartile distance. Interestingly, all tests involving Bta contain large interquartile ranges.

According to the results in Table 3 both Rno and Mmu may contain foreign examples in their datasets such that they 1) become different from each other and 2) do not fit to the general expectation. For Mmu we previously discovered that filtering their pre-miRNAs by a very simple measure (RPM > 100) leads to a 10% increase in average model accuracy for pre-miRNA detection (Saçar Demirci and Allmer, manuscript in preparation). It seems likely, that the effect of this may be even more pronounced in dependent datasets like miRNA targets since pre-miRNAs that are unlikely true lead to targets which are impossibly true. This seems to strongly affect classification accuracy in this case.

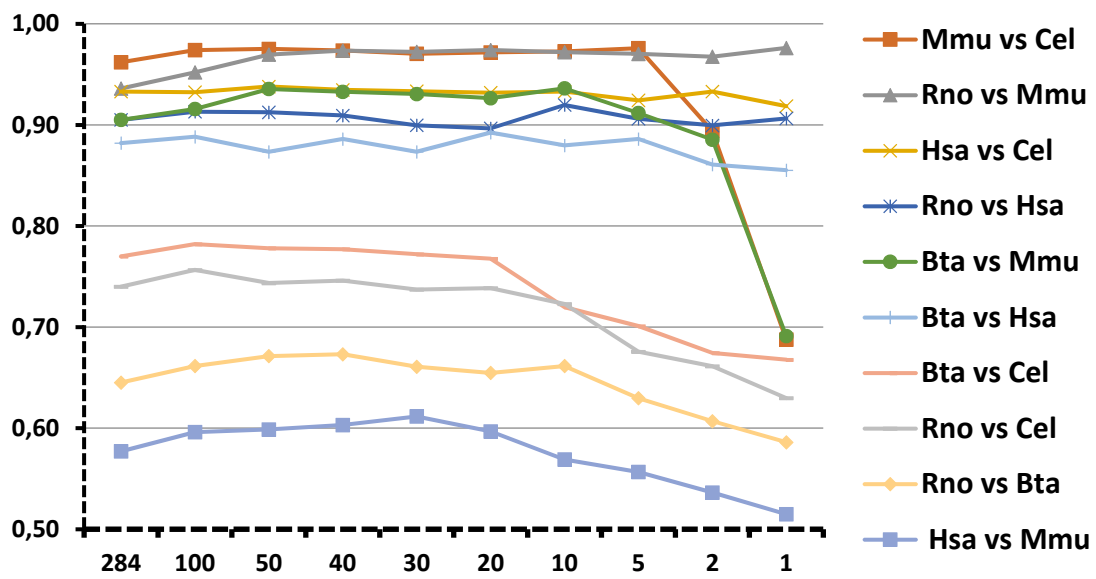


Figure 2: Average accuracy in respect to number of selected features.



Figure 3: Phylogenetic relationship among organisms and groups used in this study was established using phyloT (<http://phylo.t.biobyte.de>). Itol (<http://itol2.embl.de/>) was used to create this graph (Letunic & Bork, 2011).

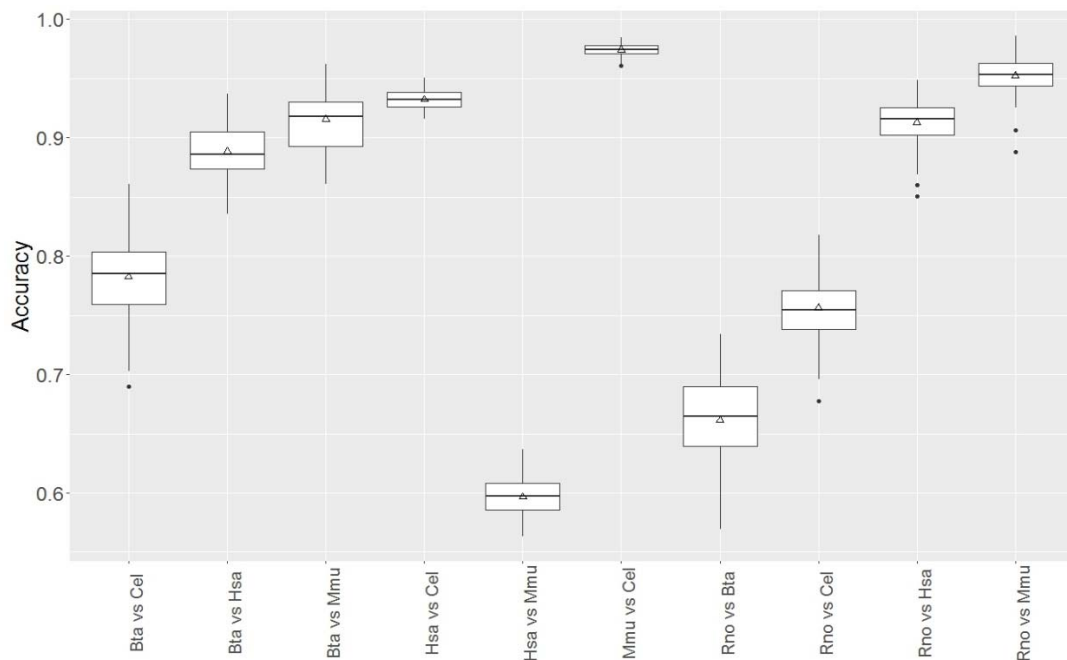


Figure 4: Accuracy distribution over 100 fold MCCV for models establishment to differentiate miRNA targets between selected species.

4 CONCLUSIONS

Machine learning has become an important tool for miRNA and miRNA target detection; however, missing negative data poses an obstacle (Allmer and Yousef, 2012). The general aim for miRNA target prediction is to determine the targets in the 3'UTRs of known genes. In this work we intended to study whether it is possible to establish machine models that can differentiate between miRNA targets from different species. A somewhat related approach previously categorized miRNAs into families, thereby, showing that miRNAs can be related (Ding et al., 2011). Contradicting this approach is that miRNAs can evolve rapidly (Liang and Li, 2009). Our aim is further supported by the finding that 3'UTRs (the most abundant targets for miRNAs) are not highly conserved (Chen and Rajewsky, 2006). Machine learning was performed using an 80/20 100-fold MCCV approach and it was shown that 100 selected features and among them generally about 30% motifs was a successful mixture for model establishment. While in general the results reflected our expectations and we can conclude that given proper examples miRNA targets can be differentiated if the phylogenetic distance is high and that it is not possible to distinguish between miRNA targets of closely related species. Additionally, we were able to show that it seems likely that among rat and mouse examples in miRTarBase there seem to be many incorrect target assignments. Nonetheless, it is our contention that miRNA targets can be distinguished between unrelated species which will be especially useful for the detection of targets in host-pathogen systems (Saçar et al., 2014; Saçar Demirci et al., 2016).

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