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Paper

Comprehensive overview and assessment of computational prediction of microRNA targets in animals

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Abstract

MicroRNAs (miRNAs) are short endogenous noncoding RNAs that bind to target mRNAs, usually resulting in degradation and translational repression. Identification of miRNA targets is crucial for deciphering functional roles of the numerous miRNAs that are rapidly generated by sequencing efforts. Computational prediction methods are widely used for high-throughput generation of putative miRNA targets. We review a comprehensive collection of 38 miRNA sequence-based computational target predictors in animals that were developed over the past decade. Our in-depth analysis considers all significant perspectives including the underlying predictive methodologies with focus on how they draw from the mechanistic basis of the miRNA–mRNA interaction. We also discuss ease of use, availability, impact of the considered predictors and the evaluation protocols that were used to assess them. We are the first to comparatively and comprehensively evaluate seven representative methods when predicting miRNA targets at the duplex and gene levels. The gene-level evaluation is based on three benchmark data sets that rely on different ways to annotate targets including biochemical assays, microarrays and pSILAC. We offer practical advice on selection of appropriate predictors according to certain properties of miRNA sequences, characteristics of a specific application and desired levels of predictive quality. We also discuss future work related to the design of new models, data quality, improved usability, need for standardized evaluation and ability to predict mRNA expression changes.

Key words: microRNA; microRNA targets; microRNA target prediction; posttranscriptional regulation; miRNA-mRNA binding

Introduction

MicroRNAs (miRNAs) are abundant and short endogenous noncoding RNAs made of 19–23 nt that bind to target mRNAs, typically resulting in degradation and translational repression of mRNAs. The fine-tuning of gene regulation in biological processes and disease pathways by these small RNAs recently attracted significant attention; the number of related articles has grown exponentially over the past decade (Supplementary Figure S1). MiRNAs are used to study signal transduction and pathogenesis of genetic [1–3], neurodegenerative [4] and metabolic diseases [5] and cancer [6, 7]. They are also used in preclinical drug development for target validation and lead optimization, and a few synthetic miRNAs entered clinical trials [8]. Development of the miRNA-directed novel therapeutics is already under way [9, 10] and miRNA-based targeting in cancer is not far behind [11, 12].

MiRNAs account for about 1% of human genes and are shown to regulate >60% of genes [13]. On average, miRNAs bind to hundreds of target sites [14], with some that have a few thousand sites [15]. The number of known miRNAs has substantially increased during the past few years, and based on release 21 of the miRBase database [16], it currently stands at >35 000 in >200 species. Unfortunately, the annotation of their targets falls

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behind as only about 1000 miRNAs (3% of known miRNAs) have validated targets. Moreover, the number of curated targets per miRNA (Supplementary Table S1) is far lower than their estimated count. Traditionally, the targets are annotated using low-throughput experimental biochemical assays including quantitative polymerase chain reaction (qPCR), luciferase assay and western blot. In recent years, a few high-throughput experimental methods to annotate miRNA targets were developed. They include microarrays and RNA sequencing that use gene expression levels and pulsed SILAC (pSILAC; stable isotope labeling by/with amino acids in cell culture) that focus on protein expression levels. These annotations are performed by assuming that miRNA targets (genes or proteins) with large reduction in expression levels in miRNA-overexpressed cells are functional (i.e. they are downregulated) [17]. One drawback of such approach to annotate miRNA targets is that it requires a threshold of the expression changes, which may vary depending on specific miRNA–mRNA pair, cell types, culture conditions, etc. Another drawback is that these experiments are done for per single miRNA and are difficult to scale to cover all known miRNAs. Lastly, these annotations are at the gene level, i.e. they indicate whether a given mRNA interacts with a given miRNA, in contrast to the duplex level, i.e. whether a given fragment on mRNA (binding site) interacts with a given miRNA. The latter is motivated by the fact that knowledge of the binding sites is important for the development of gene therapeutics [18, 19]. Cross-linking immunoprecipitation (CLIP)-based techniques attracted attention in recent years, as they can specify the sites targeted by miRNAs. However, these methods are not miRNA specific, which means that they find binding sites of the Argonaute (Ago) protein that facilitates miRNA:mRNA binding but without coupling them to specific miRNAs.

In parallel to the experimental efforts, dozens of computational miRNAs target predictors, which find targets from the mRNA and miRNA sequences, have been developed since the first method was released in 2003 [20] (Supplementary Figure S2). The underlying principle is to use data generated by (usually low-throughput) experimental methods to build predictive models, which in turn can be used to perform high-throughput predictions for specific miRNAs of interest that lack the experimental data. The results generated by these (base) predictors can be filtered or combined together by meta predictors, i.e. methods that refine predictions of the base methods such as Pio's approach and myMIR [21, 22]. However, the meta predictors often lack integration with the base predictive models (they were developed separately from the base methods and require manual collection of the predictions from the base methods) and they rely on availability of results generated by multiple base methods, which makes them more challenging to use. The targets can be also predicted computationally by ranking the gene expression or CLIP-based data, but in this case the inputs are the experimental data, which limits their applications. In this review we focus on the computational miRNAs target predictors that require only the knowledge of the miRNA and mRNA sequences (sequence-based miRNA target prediction), excluding the meta methods.

The field of sequence-based miRNA target prediction has reached maturity, as evidenced by the declining trend in the development efforts (Supplementary Figure S2). After the initial spike in 2005 when eight methods were developed, more recent years have seen on average only three new methods per year. These predictors differ on many aspects including their underlying predictive methodology (mechanistic details of miRNA–mRNA binding that they consider including use of complementarity of base pairing, site accessibility and evolutionary conservation), empirical evaluation (data sets and evaluation procedures; type of predictive model they use), usability (availability and ease of use) popularity and impact and predictive performance. Availability of many difficult-to-compare methods makes it challenging for the end users to select a proper tool and prompts the need for contributions that summarize and evaluate these methods to guide the users and to help the developers to revitalize this field. Supplementary Table S2 compares existing reviews of the miRNA target predictors based on the inclusion of discussion and analysis of the abovementioned aspects. We observe that these reviews summarized the latest miRNA target predictors at the time of their publication and compared or at least described the methodology used by these predictors. Most of these contributions also discussed availability of predictors and some aspects of their usability, focusing on the species that they were designed for. However, other important aspects of usability, such as the number of input parameters (that determines flexibility of use for an expert user), the format of the input miRNAs and genes, the ability to predict for novel miRNA sequences, the format of the outputs and the number of predicted targets (which differs substantially between methods), were omitted. They also neglected to discuss popularity and impact of the predictors and details concerning their evaluation. Only three relatively older reviews provided comparative evaluation. The first review by Rajewsky assessed nine methods on 113 experimentally annotated miRNA–target pairs, but only in *Drosophila* [23]. Review from 2006 by Sethupathy [24] used a small set of 84 annotated miRNA–target pairs and lacked assessment on the nonfunctional pairs (whether these methods can correctly recognize lack of interaction). The latest comparative review from 2009 by Alexiou [25] used 150 miRNA–target duplexes but considered only relatively old methods that were published in 2007 or earlier. Moreover, the evaluation criteria included only sensitivity and precision, which does not cover quality of prediction of the nonfunctional pairs. To summarize, prior reviews of the sequence-based miRNA target prediction methods suffer from lack of or limited and outdated empirical evaluation, inclusion of a relatively small set of predictors, lack of or shallow treatment of certain aspects, such as usability and impact of the prediction methods, evaluation procedures and practical insights for the end users and developers.

To this end, we provide a comprehensive and practical summary of this field. We introduce and discuss 38 base predictors of miRNA targets in animals including recent methods. The focus on animals is motivated by an observation that predictions of targets in plants are relatively easy and are considered a solved problem [26, 27]. We provide analysis from all key perspectives that are relevant to the end users and developers including overview of the mechanistic basis of miRNA–mRNA interaction and how this information is incorporated into the underlying predictive methodologies. We also give detailed summary of evaluation, usability and popularity/impact of the 38 predictors. As one often omitted dimension, we discuss the scope of the outputs, i.e. whether a given method provides propensity score (probability of binding) or only a binary outcome (binding versus nonbinding), and whether it predicts positions of the miRNA binding site on the target gene. We are the first to conduct an empirical comparative assessment on both low-throughput and high-throughput experimental data for the predictions at the miRNA:mRNA duplex and gene levels. We use four benchmark data sets and consider seven representative methods including recent predictors. We systematically evaluate both binary and (for the first time) real-valued propensity to compare multiple methods. Moreover, we use our in-depth analytical and empirical review to provide practical insights for the end users and developers.

Materials and methods

Benchmark data sets

There are five databases of experimentally validated and curated miRNA targets (Supplementary Table S1). Only three of them provide information necessary to characterize the miRNA:mRNA duplexes: TarBase, miRecords and miTarBase. miTarBase 4.5 stores the largest number of >5000 miRNA:target pairs [28], with large number of new data from sequencing effort in TarBase v6.0 [29]. miRecords includes 2574 interactions [30]. miR2Disease [31] and miRCancer [32] focus on selected diseases associated with miRNAs and also do not include information about miRNA:mRNA duplexes.

We developed four benchmark data sets using the miRTarBase repository, gene expression data from Gene Expression Omnibus (GEO) and pSILAC. miRTarBase provides the largest number of positive (functional) and negative (nonfunctional) miRNA:mRNA complexes; the functional miRNA–mRNA interactions are defined as those where mRNA is downregulated by the corresponding miRNA. GEO is the largest source of microarray, sequencing and other forms of high-throughput genomics data [33]. pSILAC is a technique for quantitative proteomics [34]. Our data sets cover human and mouse, which is motivated by research interests in using miRNAs in human health–related applications [35, 36] and our objective to include the largest possible number of predictors, i.e. relatively few methods work on other species.

The first data set, called TEST_duplex, is used to assess the target site prediction at the duplex level. We selected targets that were validated by at least one of the low-throughput experimental methods, which are considered as strong evidence: qPCR, luciferase assay or western blot. We focused on targets that were released recently to limit overlap between our benchmark data and data used to develop the evaluated predictors. The functional targets deposited to miRTarBase after 2012 (after the newest method included in our evaluation was published) and all nonfunctional duplexes from human and mouse were included; we used all nonfunctional targets because of their small number. The second, TEST_gene data set focuses on the evaluation at the gene level. We selected miRNAs that have both functional and nonfunctional genes in miRTarBase and for which the functional genes were validated after 2012.

Furthermore, we extend our evaluation to analyze whether the current methods are capable of predicting at the cell level using two additional data sets that rely on the annotations from the high-throughput methods. TEST_geo data set is based on results from three microarray-based experiments: GSE6838, GSE7864 and GSE8501. The interactions for 25 miRNAs were annotated the contrasting expression arrays before miRNA transfection and at 24 h after miRNA mimics were transfected [37–39]. As recommended in [40, 41], we remove the genes for which the expression magnitudes are below the median in the control transfection experiments. TEST_psilac data set was originally developed in a proteomic study that used pSILAC technique [34, 42]. Previous studies assume that genes that are more repressed (characterized by higher drop in the expression levels) are more likely to be targeted by the transfected miRNA. These studies use a certain fraction of the genes with the highest magnitude of the change in the expression levels (repressed genes) as functional and the same fraction of the genes for which expression levels have increased by the largest margin (overexpressed genes) as nonfunctional [40, 43]. Instead of using an arbitrary fraction value to define the functional and nonfunctional targets, we vary this value between 1% and 50%. Detailed summary of the four data sets is shown in the Supplementary Table S3. The TEST_duplex and TEST_gene data sets are given in the Supplementary Tables S4 and S5, respectively.

The comprehensiveness of our tests stems from the fact that we consider targets as gene segments (TEST_duplex data set), genes (TEST_gene and TEST_geo data sets) and proteins (TEST_psilac data set). We also use different source of information that is used to perform annotations including low-throughput assays (TEST_duplex and TEST_gene data sets), microarrays (TEST_geo data set) and pSILAC (TEST_psilac data set).

Considered miRNA target predictors

We selected several representative predictors for the empirical evaluation. The selected methods have to be conveniently accessible to the end users via a web server or a precomputed database. They also have to cover human and mouse, predict target sites (to perform evaluation at the duplex level) and provide propensity (probability) of the interaction. Using these filters we selected eight methods (see Supplementary Table S6). We use their latest versions of these methods, except for PicTar2, which is substantially different from PicTar and no longer qualifies as a sequence-based predictor. PicTar 2005 was first published in 2005; five methods including TargetScan 6.2, miRanda 2010, EIMMo3, miREE and mirTarget2 v4 were proposed or updated between 2010 and 2012; and two in 2013: DIANA-microT-CDS and miRmap v1.1. We excluded miREE from the evaluation because this method did not predict any targets on our TEST_duplex and TEST_gene data sets. The remaining seven methods use a diverse set of predictive models, with four that use heuristic scoring functions and three that use the machine learning models including Bayesian classifier, support vector machine (SVM) and regression. miRmap was built based on gene expression data, while the other methods were derived based on the low-throughput experimentally validated data. We collected predictions for these methods using either their online web servers or downloadable precomputed predictions. We recorded their predicted binding targets (sequences or positions) and the corresponding propensities.

Criteria for empirical evaluation

We used a comprehensive set of evaluation measures to assess the predictions of the miRNA:target duplexes and miRNA–gene pairs. Each prediction takes two forms: binary value that indicates whether a given duplex or miRNA–gene pair is predicted to be functional; and the real-valued probability (propensity) of a given predicted interaction.

The binary predictions were assessed using the following seven measures:

$$\text{Sensitivity} = \frac{TP}{TP + FN},\ \text{Specificity} = \frac{TN}{TN + FP},\ \text{Precision} = \frac{TP}{TP + FP},$$

$$\text{MCC} = \frac{TP \times TN - FP \times FN}{\sqrt{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}},$$

$$\text{SNR\_functional} = \frac{TP}{FP},\ \text{SNR\_non\_functional} = \frac{TN}{FN},$$

$$\text{PNR} = \frac{TP + FP}{TP + FN} = \frac{\text{Sensitivity}}{\text{Precision}}$$

where true positives (TP) and true negatives (TN) are the counts of correctly predicted functional and nonfunctional miRNA targets, respectively, and false positives (FP) and false negatives

(FN) are the counts of incorrectly predicted functional and nonfunctional miRNA targets, respectively. The values of the Matthews Correlation Coefficient (MCC) range between −1 and 1, with 0 for random predictions and higher values denoting more accurate predictions. MCC provides a robust measurement for skewed data sets (when number of positive and negative outcomes in unbalanced), which is the case with our TEST_duplex data set. Signal-to-Noise Ratio (SNR) of correctly over incorrectly predicted functional targets was calculated in several prior works [20, 44–47]. We computed the SNR of predicted functional (SNR+) and also nonfunctional samples (SNR−) to provide a complete set of measures. Given the skewed counts of native (true) functional and nonfunctional samples in our data sets, we normalized the SNR values as follows:

$$\mathrm{SNR+duplex}=\frac{\mathrm{TP\_duplex}}{\mathrm{FP\_duplex}}\Big/\frac{\mathrm{P\_duplex}}{\mathrm{N\_duplex}},\ \mathrm{SNR-duplex}=\frac{\mathrm{TN\_duplex}}{\mathrm{FN\_duplex}}\Big/\frac{\mathrm{N\_duplex}}{\mathrm{P\_duplex}},$$

$$\mathrm{SNR+gene}=\frac{\mathrm{TP\_gene}}{\mathrm{FP\_gene}}\Big/\frac{\mathrm{P\_gene}}{\mathrm{N\_gene}},\ \mathrm{SNR-gene}=\frac{\mathrm{TN\_gene}}{\mathrm{FN\_gene}}\Big/\frac{\mathrm{N\_gene}}{\mathrm{P\_gene}}$$

where P_duplex (P_gene) and N_duplex (N_gene) are the numbers of native (true) functional and nonfunctional duplexes (genes) in the TEST_duplex (TEST_gene) data set. The overall count of predicted functional targets is assessed using Predicted-to-Native positive Ratio (PNR) = predicted_functional_count/true_functional_count. PNR indicates whether a given predictor overpredicts (PNR value >1) or underpredicts (PNR value <1) the number of functional miRNA targets.

The real-valued propensities were assessed using the receiver operating characteristic (ROC) curve, which represents relation between true-positive rates (TPR) = TP/(TP + FN) and false-positive rates (FPR) = FP/(FP + TN). The ROC curves reflect a trade-off between sensitivity and specificity, providing comprehensive information about the predictive performance. We compute the area under the ROC curve (AUC) that ranges between 0 (for a method that does not predict TP) and 1 (for a perfect predictor), with 0.5 denoting a random predictor.

Except for the PNR and SNR−, which we introduced, and the normalization of the SNR+ and SNR− values that is motivated by the unbalanced nature of the benchmark data sets, the other criteria were used to evaluate some of the prior predictors [48–53] (see column 'Criteria' in Table 2).

We also evaluate statistical significance of differences in predictive performance between predictors. We randomly choose 50% of a given data set, calculate the predictive performance and repeat this 10 times. The corresponding 10 pairs of results (to compare a given pair of predictors) are evaluated with the student's *t*-test if distributions are normal; otherwise we use the Mann–Whitney test. The distribution type is verified using the Anderson–Darling test with the *P*-value of 0.05.

Results

Considered miRNA target predictors

We consider 38 sequence-based methods, from the earliest predictor that was published in 2003 to the latest method that was released in 2013; chronological list of methods is shown in Table 1. We exclude the meta methods (because they are inconvenient to use and require availability of results from base methods) and approaches that rely on the experimental data. Most of the miRNA target predictors were developed by different research groups, with several groups that continue maintaining and updating their algorithms. Cohen's group at EMBL proposed the first miRNA target predictor in 2003 [20] and updated it in 2005 [62]. TargetScan and TargetScanS were developed by Bartel at MIT and Burge at Cambridge [13, 39, 44, 56]. Another popular tool, DIANA-microT, which was created by Hatzigeorgiou group, has been recently updated to version 5.0 [77–79]. Rajewsky's lab published their predictor PicTar in 2005 and updated it in 2011 [46, 80].

Predictive methodologies and mechanistic basis of miRNA–mRNA interaction

Table 1 summarizes types of predictive models and the underlying details of the miRNA–mRNA interactions that they use to predict miRNA targets. There are two categories of predictive models: heuristic and empirical. The heuristic models use screening algorithms that search positions along the mRNA sequence and scoring functions that filter targets by combining values of several inputs in an *ad hoc* manner. Early predictors applied heuristic approaches owing to the lack of sufficient amount of data to build the empirical knowledge-based models. Even today the scoring function-based designs are dominant (19 of 38 methods) because of their easy setup, flexibility to integrate different types of inputs and computational efficiency. The empirical models are inferred from a training data set. Given the success of machine learning-based models in bioinformatics [81, 82] and growing size of the experimental data, since 2006 progressively more predictors use empirical machine learning models including SVMs, decisions trees and artificial neural networks (ANNs).

The predictive models use inputs that are derived from the knowledge of mechanistic details of the miRNA–mRNA interactions. The most commonly used predictive input is the complementarity of the base pairing between miRNA and mRNA. In contrast to the near-perfect base pairing in plants [26], animal miRNAs usually bind mRNAs with only some positions that are paired [83]. Complementarity of the base pairing in the seed region (the first eight nucleotides at the 5′ end of miRNAs) is particularly important; only six methods did not consider it. To compare, 15 methods did not consider complementarity in the nonseed region. The major types of complementarity in the seed include 6-mer (six consecutive matches between second and seventh positions from the 5′ end of miRNA), 7-mer-A1 (extends 6-mer with an adenine (A) nucleotide at the first position of target 3′ end), 7-mer-m8 (seven consecutive matches from second to eighth position of miRNA) and 8-mer (combines 7-mer-m8 and 7-mer-A1). Some methods consider binding of the first eight nucleotides as important but do not restrict it to particular seed types. Moreover, several predictors (HuMiTar [48], TargetMiner [49], MultiMiTar [70], miREE [71] and SuperMirTar [74]) also suggest specific positions that are more useful for the prediction. These methods, except for HuMiTar, use machine learning models and empirical feature selection to find these positions. One other exception is that TargetBoost [61], RNA22 [15] and SVMicrO [50] use patterns of complementarity generated from native miRNA:mRNA complexes, rather than focusing on the seed types.

The site accessibility and evolutionary conservation inputs are used to increase specificity. The accessibility is relevant because miRNA:mRNA interaction requires binding of a relatively large RNA-induced silencing complex [84]. This input is quantified with content of adenine and uracil nucleotides (AU content) and free energy that estimates stability of the mRNA sequences. Most target predictors use existing software, like Vienna RNA

Table 1. Methodologies and the corresponding mechanistic basis of miRNA–mRNA interaction used by the miRNA target predictors

Predictor	Reference	Year published	Model type	Complementarity		Site accessibility		Conservation	Multiple sites	Features	
				Seed	Nonseed	Free energy	AU %			Count	Selection
Stark *et al.*	[20]	2003	Screening	1–8	miRNA size+5	mFold	✗	a d	✓	✗	✗
TargetScan	[44]	2003	Score	7mer-m8	to 1st mismatch	Vienna RNA	✓	m r z	✗	✗	✗
DIANA-microT	[54]	2004	Score	✗	38 nt	✓		m	✓	✗	✗
RNAhybrid	[47]	2004	Score	6mer	✗	RNAhybrid	✗	a d	✓	✗	✗
miRanda	[55]	2004	Score	7mer-m8	✗	Vienna RNA	✗	f m r	✓	✗	✗
Rajewsky's	[23]	2004	Score	1–8	✗	mFold	✗	d	✗	✗	✗
TargetScanS	[56]	2005	Score	6mer	✗	✗	✗	c g h m r	✗	✗	✗
Robins	[57]	2005	Score	2–8	✗	Vienna RNA	✗	✗	✗	✗	✗
Xie *et al.*	[58]	2005	Score	8mer	✗	✗	✗	g h m r	✓	✗	✗
PicTar	[46]	2005	Score	7mer-A1, 7mer-m8	Remaining	mFold	✗	d	✗	✗	✗
MovingTarget	[59]	2005	Screening	1–8	50 nt	DINAMelt	✗	d	✓	✗	✗
MicroInspector	[60]	2005	Score	7mer-A1, 7mer-m8	✗	Vienna RNA	✗	✗	✗	✗	✗
TargetBoost	[61]	2005	GP	pattern	30 nt	mFold	✗	✗	✗	✓	✗
Stark *et al.*	[62]	2005	Score	6mer	10th nt to end	RNAhybrid	✗	d	✓	✗	✗
miTarget	[63]	2006	SVM	2–7	20 nt	Vienna RNA	✓	✗	✗	15	Wrapper
RNA22	[15]	2006	Score	✗	Pattern	✗	✗	✗	✓	✗	✗
MicroTar	[64]	2006	Score	7mer-A1, 7mer-m8	✗	Vienna RNA	✗	✗	✓	✗	✗
EIMMo	[65]	2007	Bayesian	7mer-A1, 7mer-m8	✗	✗	✗	✓	✗	✓	✗
STarMir	[66]	2007	Score	✗	miRNA size	sFold	✗	✗	✗	✗	✗
PITA	[67]	2007	Score	6mer	✗	Vienna RNA	✗	✗	✓	✗	✗
TargetRank	[68]	2007	Score	6mer	✗	✗	✓	✓	✓	✗	✗
MirTarget2	[43]	2008	SVM	6mer	✗	Vienna RNA	✗	✗	✗	6	Filter
HuMiTar	[48]	2008	Score	6mer	9–13, 14–20 nt	✗	✗	✗	✗	✗	✗
TargetMiner	[49]	2009	SVM	6mer	13–16 nt	✓	✓	✓	✓	30	Filter
TargetSpy	[52]	2010	DS	✗	All	Vienna RNA	✗	✗	✗	7	Filter
Mtar	[53]	2010	ANN	6mer	Remaining	Vienna RNA	✗	✗	✗	16	✗
mirSVR	[40]	2010	Score	2–7	✗	miRNAbind	✓	✓	✗	✗	✗
SVMicrO	[50]	2010	SVM	5 patterns	Remaining	Vienna RNA	✓	✓	✗	39	Wrapper
RepTar	[69]	2010	Screening	6mer	Remaining	Vienna RNA	✗	✓	✓	✗	✗
PACMIT	[51]	2011	Screening	✗	Remaining	Vienna RNA	✗	✗	✓	✗	✗
MultiMiTar	[70]	2011	SVM	6mer	13–16 nt	✗	✓	✗	✓	39	Filter
miREE	[71]	2011	SVM	1–8	13–16 nt, remain	Vienna RNA	✓	✗	✗	25	Filter
miRcode	[72]	2012	Screening	7mer-A1, 7mer-m8	✗	✗	✗	P M other V	✓	✗	✗
miRmap	[41]	2012	Regression	6mer	Remaining	Vienna RNA	✓	M	✓	12	Filter
HomoTarget	[73]	2012	ANN	1–8	Remaining	✓	✗	✗	✗	12	Filter
SuperMirTar	[74]	2013	Graph	6mer	12–17 nt	RNAhybrid	✓	✗	✗	✗	✗
Fujiwara's	[75]	2013	Cis-element	✗	✗	✗	✗	✗	✗	✗	✗
MIRZA	[76]	2013	Bayesian	1–8	Remaining	✓	✗	✗	✗	✗	✗

We summarize key aspects including model type, region that is searched to predict targets and inclusion of several mechanistic properties that are known to provide useful inputs for prediction, such as complementarity between miRNA and mRNA, site accessibility and conservation across species; ✗ means that a given aspect was irrelevant or not considered. Predictors are sorted in the chronological order. 'Model type' describes type of predictive model type including screening of the mRNA sequence, heuristic scoring function and empirically designed genetic programming (GP), support vector machine (SVM), decision stump (DS) and artificial neural network (ANN) models. 'Complementarity' section indicates positions for which complementarity of base pairs between miRNA and mRNA was explored in the seed (first eight positions on the miRNA) and nonseed regions. Four common seed types are 6-mer, 7-mer-A1, 7-mer-m8 and 8-mer; they have consecutive complementary base pairs on these positions. '1–8', '2–8', etc. annotations mean that these do not have to be consecutive complementary base pairs. Nonseed denotes the center and 3′ end of the miRNA region where e.g. 38 nt means the size of the targets is up to 38 nt; 14–20 nt indicate the nonseed regions is considered from the 14**th to 20th** nucleotide; 'remaining' refers the region from the end of the seed to the end of the miRNA. 'Site accessibility' describes inclusion of two aspects: AU content around the targets and free energy. If free energy is used, then the name of the package used to calculate it is given (if known), otherwise ✓ is used. 'Conservation' column indicates species that were used in calculation of conservation: anopheles (a), chicken (c), drosophila (d), fungi (f), dog (g), human (h), mouse (m), nematode (n), rat (r), zebra fish (z), primate (P), mammal (M) and vertebrate (V). If conservation is used but species are unknown, then ✓ is used. Methods that consider prediction of multiple sites on the same gene are annotated with ✓ in the 'Multiple sites' column. For machine learning methods, the 'features' column indicates number of used features and whether and what feature selection approach was used; ✓ denotes the features are used but the count is unknown.

package [85], mFold [86], DINAMelt [87] and sFold [88], to calculate the free energy. Authors of RNAhybrid claim that their own approach prevents intramolecular base pairing and bulge loops, which leads to improved estimates of the free energy [47]; this approach was also used in the predictor by Stark et al. [62] and in SuperMirTar [74]. Most predictors calculate the free energy of the miRNA–target duplexes. However, several methods (MicroTar [64], STarMir [67], PITA [66], TargetMiner [49], SVMicrO [50], PACMIT [51] and miREE [71]) calculate arguably more relevant relative energy, which is the hybridization energy gained by miRNA:mRNA binding minus the disruption energy lost by opening up the local mRNA structure of the target. Several studies found that enriched AU content in mRNA 3′ untranslated regions (UTRs) is important for interaction with miRNAs [89–91]. This was exploited in 2003 in TargetScan, even before experimental data to that effect was published [39]. Since then several methods have used this information (see 'AU %' column in Table 1). Use of the evolutionary conservation of miRNA targets is motivated by a premise that 'similar' species should share common miRNAs and their targets. However, this leads to

Table 2. Protocols for evaluation of the miRNA target predictors

Predictor	Reference	Benchmark data sets				Evaluation procedures			
		Species	Number of training duplexes	Number of test duplexes	Nonfunctional samples	Number validated targets	Criteria	Statistical test	Functional analysis
Stark *et al.*	[20]	d	✗	5+	Shuffled miRNA	6	SNR, conservation	✓	✓
TargetScan	[44]	h m p	✗	Gene level	Shuffled miRNA	11	FPR, SNR	✗	✓
DIANA-microT	[54]	h	✗	11+	Shuffled miRNA	0	SNR	✗	✗
RNAhybrid	[47]	d	✗	11+	Shuffled miRNA	0	SNR	✓	✗
miRanda	[55]	h z	✗	8+	Shuffled miRNA	0	FPR	✗	✗
Rajewsky's	[23]	d	25	Gene level	Random mRNA	0	FPR	✗	✗
TargetScanS	[56]	V	✗	✗	Shuffled miRNA	0	SNR	✗	✓
Robins	[57]	d	✗	✗	✗	10	✗	✓	✗
Xie *et al.*	[58]	h	✗	✗	✗	12	✗	✗	✗
PicTar	[46]	d	✗	19+	Shuffled miRNA	0	SNR, sensitivity	✗	✓
MovingTarget	[59]	d	✗	✗	✗	3	✗	✗	✗
MicroInspector	[60]	d	✗	✗	✗	0	✗	✗	✗
TargetBoost	[61]	d n	36+, 3000–	✗	Random mRNA	0	AUC	✓	✗
EMBL	[62]	d	✗	Gene level	Shuffled miRNA	8	✗	✓	✓
miTarget(43)	[63]	h	152+, 246–	Same with training	4-mer on nonpositives	0	AUC	✗	✓
RNA22	[15]	d h n m	✗	21+	Shuffled miRNA	168	FPR	✗	✗
MicroTar	[64]	d m n	✗	63, 13 and 43+	✗	0	sensitivity	✗	✗
EIMMo	[65]	d n z M	✗	120 in all	Validated	0	sensitivity, specificity	✗	✗
STarMir	[66]	d n	✗	39+, 12–	Validated	0	FPR, SNR	✗	✗
PITA	[67]	d	✗	123+, 67–	Validated	0	AUC	✗	✗
TargetRank	[68]	V	✗	✗	✗	0	✗	✗	✗
MirTarget2	[43]	c g h m r	✓	✓	Validated	0	AUC	✗	✗
HuMiTar	[48]	h	66 in all	39 and 190 in all	Validated	0	AUC, SNR	✗	✗
TargetMiner	[49]	h	289+, 100–	187+, 59–	Microarray+validated	0	MCC, ACA	✗	✗
TargetSpy	[52]	c d h m r	3872+, 4540–	61+, 59–/102+, 88–	pSILAC+validated	0	AUC	✗	✗
Mtar	[53]	h	150+, 200–	190+, 200–	Validated	0	AUC	✗	✗
mirSVR	[40]	h	Gene level	Gene level	microarray+CLIP	0	AUC	✗	✗
SVMicrO	[50]	h m r	324+, 3492–	Gene level	Microarray	0	AUC	✗	✗
RepTar	[69]	h m v	197 and 22 in all	Same with training	Validated	0	precision, accuracy	✓	✗
PACMIT	[51]	d h	137+, 83–/2406+, 13400–	Same with training	pSILAC+validated	0	specificity and pROC	✓	✗
MultiMiTar	[70]	h	289+, 289–	187+, 57–	pSILAC+validated	0	MCC, ACA	✗	✗
miREE	[71]	d h m n r v z	324+, 351	2 new data sets	pSILAC+PAR-CLIP+ validated	0	pROC	✓	✗
miRcode	[72]	V	✗	✗	✗	0	✗	✗	✓
miRmap	[41]	h m	Gene level	Same with training	Microarray; CLIP	0	✗	✗	✗
HomoTarget	[73]	h	112 pos + 313 neg	Same with training	Validated	0	AUC	✗	✗
SuperMirTar	[74]	h m	2860 human, 582 mouse	674+, 15132–	pSILAC+valiated	0	AUC	✗	✗
Fujiwara's	[75]	h	✗	155+	Validated	0	pROC	✗	✗
MIRZA	[76]	all available	Gene level	Same with training	Ago2-CLIP	0	Sensitivity	✗	✗

We describe the benchmark data sets used to design and test the predictors including the target 'species', size of the training and test data sets and the source of the nonfunctional samples. ✗ means that a given aspect was irrelevant or not considered. The 'species' are anopheles (a), chicken (c), drosophila (d), fungi (f), dog (g), human (h), mouse (m), nematode (n), rat (r), virus (v), zebra fish (z), mammals (M) and vertebrates (V). 'Number training/test duplexes' columns give the number of functional (+) and nonfunctional (–) samples if they were provided; otherwise ✓is used. The 'nonfunctional samples' column describes the sources of the nonfunctional examples; they include either targets with validated lack of interaction with a given miRNA or artificially generated (using shuffling or randomization) samples. We also describe procedures used to assess the predictive performance of the predictors. This includes the number of the experimentally validated targets used, criteria used to measure the performance and whether statistical tests and functional analysis were performed. 'Number of validated targets' shows the number of experimentally tested predicted targets. 'Criteria' lists the criteria used to assess the programs: signal-to-noise ratio (SNR), false positive rate (FPR), area under ROC curve (AUC), Matthews correlation coefficient (MCC) and average class-wise accuracy (ACA). Methods for which predictions were assessed with statistical tests of significance and for which functional analysis was performed are indicated with ✓ in the 'statistic test' and 'function' columns, respectively.

omission of the nonconserved targets [64, 92]. The value of the inclusion of the target conservation remains an open question; Table 1 reveals that conservation is used less frequently in recent years. Still, methods that search for targets in long coding DNA segments (CDSs) use conservation to improve specificity [59, 72, 93, 94]. Based on an observation that targeting of multiple sites enhances the mRNA regulation [95, 96], 17 of the 38 methods increase the propensity of binding to a target gene with multiple predicted sites (see 'Multiple sites' column in Table 1).

The machine learning models often use empirical approaches to select inputs (features) that are relevant to the prediction of miRNA targets. Table 1 shows that the count of the selected features ranges from a few to a few dozen; these features quantify specific aspects related to the complementarity, accessibility and conservation. The considered feature-selection approaches include wrapper- and filter-based methods. The former approach searches for the best subset of features to maximize predictive performance of a given machine learning model. Filters rank features according to a metric, like F-score or correlation, and select a predefined number of the top-ranked features.

Evaluation protocols

Benchmark data sets used to develop and test the predictors and the corresponding evaluation procedures are summarized in Table 2. Many early methods were designed/evaluated using data only from *Drosophila* owing to limited availability of validated miRNA targets. However, even some early predictors (TargetScan [33], DIANA-microT [44], miRanda [97] and TargetScanS [56]) considered higher eukaryotes. More recent methods generally cover more species. Interestingly, in 14 cases predictors were validated on test data sets but there was no mention about data being used to design these predictive models. This may mean that the test data was used in the design, e.g. to set thresholds and parameters. HuMiTar was the first method that was properly tested on an independent (from the training set) data set [48]. Even with the currently available relatively large number of validated miRNA targets, only a few recent predictors (TargetMiner [49], TargetSpy [52], Mtar [53], MultiMiTar [70] and miREE [71]) were trained and tested on different (independent) data sets. Moreover, the sizes of some training data sets are relatively small (a few dozen samples) and some data sets are unbalanced and have more artificial nonfunctional targets than the functional targets; some data sets use only a few validated nonfunctional targets. A particularly challenging aspect is a low number of experimentally validated nonfunctional samples, i.e. mRNA validated not to interact with a given miRNA. Several early methods used artificial nonfunctional targets created by either shuffling miRNAs sequences or by randomization of mRNAs; these approaches were criticized to generate unrealistic samples [49]. More recent attempts scan the mRNA transcripts where validated target sites or Ago-binding sites are masked and use the target segments with at least 4-mer matches in the seed region or one mismatch or G:U wobble in the 6-mer seed as the nonfunctional samples [40, 53, 63]. This approach assumes that the knowledge of functional targets or Ago-binding sites is complete, while in fact these computationally generated nonfunctional miRNA–mRNA pairs could be functional. Some recent methods label overexpressed genes when particular miRNA mimics are added to cells as nonfunctional, but data from this limited number of miRNAs may be biased.

These various attempts to generate the benchmark data sets may result in mislabeling, overfitting the training data sets and generation of unrealistic (possibly inflated) evaluation of predictive performance.

We also analyze the evaluation procedures. The early predictors were evaluated primarily based on SNR between the number of predicted targets in functional genes and in true or artificial nonfunctional genes. PicTar was the first to report sensitivity, based on only 19 native targets. TargetBoost and miTarget were the first to use more informative ROC curves, but with the caveat of using artificial nonfunctional targets. The criteria used to evaluate predictive quality vary widely between methods. Some measures are biased by the composition of the data set (e.g. accuracy and precision) and provide incomplete picture (e.g. sensitivity without specificity and vice versa). This makes comparisons across predictors virtually impossible. The standards to compare between methods are also relatively low, as in most cases evaluation did not include statistical tests. On the positive side, the assessment of several methods included experimental validation of targets. The authors of RNA22 method performed a large-scale validation and claimed that 168 of 226 tested targets were repressed; however, they did not find whether these targets were bound by the specific miRNAs. Some primarily older methods also included functional analysis of the predicted targets.

Usability and impact

Table 3 shows that miRNA target predictors are available to the end users as web servers, stand-alone packages, precomputed data sets and upon request. The 21 methods that are provided as web servers are convenient for *ad hoc* (occasional) users. The 13 stand-alone packages are suitable for users who anticipate a high-throughput use and/or who would like to include them into their local software platforms; most of these are also available as the web servers. The convenient to collect precomputed results are provided for 10 methods. However, these predictions may not be updated timely and do not include results for novel miRNAs.

The ease of use is affected by the use and number of parameters, scope of predictions, format of inputs and ability to predict for novel miRNAs. The prediction methods rely on parameters that can be used to control how prediction is performed, e.g. the seed size, the number of allowed guanine–uracil wobbles and mismatches, selection of mRNA regions that are searched and the cutoffs for free energy and predicted propensity score. These parameters are usually set based on experience of the designer or user of a given method, or are optimized empirically using a data set. Eleven methods hardcode and hide these parameters from the users, which arguably makes them easier to use but also reduces ability of the end users to tune the models for specific needs or projects. RNAhybrid [47] offers eight (the most) parameters for tuning; RepTar and PITA [67, 69] have seven and five parameters, respectively; and eight predictors allow adjusting between one and four parameters. Importantly, these predictors provide default values for the parameters, so they can be seamlessly used even by layman users.

A ‘user-friendly’ method should allow predicting a wide range of species and target types. Most of the early methods only allow predictions in the 3′UTRs, except for RNAhybrid [47], miRanda [98], DIANA-microT-CDS [79] and PACMIT-CDS [94], that also search coding DNA sequences (CDSs) and TargetScanS [56] and Xie’s method [58] that consider open reading frames (ORFs) and promoters, respectively. As more miRNA targets were discovered beyond the 3′UTRs [91, 99], several newer programs (RNA22 [15], STarMir [66], Mtar [53] and miRcode [72]) predict in the 3′UTRs, CDSs and 5′UTRs. A few methods (RNAhybrid [47], MicroInspector [60], MicroTar [64] and MIRZA [100]) do not limit species for which they predict. They accept target genes as RNA sequences or provide stand-alone packages where users can prepare their own mRNA database. Most of the other predictors are constrained to human, mouse, fly and worm. The latter two were the first two species that were used to study miRNA targets. Seven methods consider a more restrictive set

Table 3. Usability and impact of the miRNA target predictors

Predictor	Availability		Ease of use						Impact/popularity		
	Type	URL	Number para-meters	Target region	Coveredspecies	Number targets	Format of gene	New miRNA	High-lighted	consi-dered	Number citations
Stark *et al.*	✗	✗	~	3′UTR	d	~	~	✗	0	4	34.4
TargetScan	s ws	http://www.targetscan.org/	3	3′UTR	d h m n z	A few	Name	✓	3	14	429.5
DIANA-microT	ws	http:http://diana.pcbi.upenn.edu/DIANA-microT	1	3′UTR	d h m n r t	A few	Name	✓	2	14	38.4
RNAhybrid	s ws	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	8	3′UTR, CDS	Any	Dozens	Sequence	✓	2	12	69.3
miRanda	s ws p	http://www.microrna.org/microrna/home.do	0	3′UTR, CDS	d h m r n	1000s*	None	✗	0	15	104.8
Rajewsky's	✗	✗	~	3′UTR	d	~	~	~	0	2	18.5
TargetScanS	s ws	**http://genes.mit.edu/tscan/targetscanS2005.html**	0	3′UTR, ORFs	d m n other V	100 s*	Name	✗	4	10	429.5
Robins	✗	✗	~	3′UTR	~	~	~	~	0	2	14.6
Xie *et al.*	✗	✗	~	Promoters and 3′UTR	~	~	~	~	0	2	124.8
PicTar	ws p	http://pictar.mdc-berlin.de/	0	3′UTR	d h m n	Dozens	Name	✗	1	16	26.9
MovingTarget	ur	✗	~	3′UTR	~	~	~	~	0	5	5.7
MicroInspector	ws	http://bioinfo.uni-plovdiv.bg/microinspector/	2	3′UTR	Any	A few	Either	✓	0	3	13.0
TargetBoost	demo	**http://www.interagon.com/demos.html**	~	3′UTR	n	~	~	~	0	8	7.9
Stark *et al.*	p	http://mirnas.russelllab.org/	~	3′UTR	~	100 s*	~	~	1	5	67.8
miTarget	✗	**http://cbit.snu.ac.kr/miTarget**	~	3′UTR	~	~	~	~	0	9	10.4
RNA22	ws p	https://cm.jefferson.edu/rna22v1.0/	4	3′UTR, CDS	d h m n	1000s*	Name	✗	0	13	80.8
MicroTar	s	http://tiger.dbs.nus.edu.sg/microtar/	0	3′UTR	any	~	~	✓	0	3	5.6
EIMMo	ws p	http://www.mirz.unibas.ch/ElMMo3/	0	3′UTR	d h m r n z	a few	Name	✗	1	6	17.4
STarMir	ws	http://sfold.wadsworth.org/cgi-bin/starmir.pl	0	3′UTR, CDS, 5′UTR	h m	dozens	Either	✓	0	1	28.3
PITA	s ws	http://genie.weizmann.ac.il/pubs/mir07/index.html	5	3′UTR	d h m n	a few	Either	✓	1	6	97.6
TargetRank	ws	http://hollywood.mit.edu/targetrank/	0	3′UTR	h m	100 s*	None	✗	0	1	22.9
MirTarget2	ws p	http://mirdb.org/miRDB/	0	3′UTR	c g h m r	a few	Name	✗	0	4	26.4
HuMiTar	ur	✗	3	3′UTR	h	~	Sequence	✓	0	1	2.2
TargetMiner	ws p	http://www.isical.ac.in/~bioinfo_miu/targetminer20.htm	0	3′UTR	h	a few	Name	✗	0	2	8.7
TargetSpy	s ws	http://www.targetspy.org/	2	3′UTR	c d h m r	a few	Name	✗	0	1	7.8
Mtar	✗	✗	~	3′UTR, CDS, 5′UTR	~	~	~	~	0	1	4.0
mirSVR	✗	✗	~	3′UTR	~	~	~	~	0	1	52.0
SVMicrO	s	http://compgenomics.utsa.edu/svmicro.html	~	3′UTR	h m r	~	~	~	0	1	4.3
RepTar	s ws p	http://bioinformatics.ekmd.huji.ac.il/reptar/	7	3′UTR	h m	a few	Name	✗	0	0	2.5
PACMIT	✗	✗	~	3′UTR	~	~	~	~	0	0	7.3
MultiMiTar	s ws	http://www.isical.ac.in/~bioinfo_miu/multimitar.htm	0	3′UTR	h	a few	Either	✓	0	0	3.3
miREE	s	http://didattica-online.polito.it/eda/miREE/	0	3′UTR	d h m n r z	dozens	Either	✓	0	0	1.0
miRcode	ws p	http://www.mircode.org	3	3′UTR	h	a few	Name	✗	0	1	5.3
miRmap	s ws p	http://mirmap.ezlab.org/	4	3′UTR, CDS, 5′UTR	c e h m o r w z	a few	Either	✓	0	0	4.0
HomeTarget	✗	✗	~	3′UTR	~	~	~	~	0	0	0.5
SuperMirTar	✗	✗	~	3′UTR	~	~	~	~	0	0	0.0
Fujiwara's	ur	✗	~	3′UTR	~	~	~	~	0	0	0.0
MIRZA	s ws	http://www.clipz.unibas.ch/index.php?r=tools/sub/mirza	0	3′UTR	Any	s few	Sequence	✓	0	0	14.0

We summarize availability, ease of use and impact/popularity. ✗ means that a given aspect was missing. ~ denotes unknown, as the information was not available in the paper or on the web server. 'Availability' focuses on type of implementation available to the end user: stand-alone (s), web server (ws), precomputed results (p) and upon request (ur), and provides the corresponding URLs. The links shown in bold font did not work. 'Ease of use' covers aspects related to the scope of a given method and ease to run it including the number of input parameters of the corresponding web servers, the targets regions and species that can be predicted, the approximate number of predicted targets, the format in which the searched genes are provided and the ability to predict for new miRNAs. 'Target region' indicates where a given method searches for targets: untranslated region (UTR), coding DNA segment (CDS) and open reading frame (ORF). The covered species are chicken (c), drosophila (d), chimpanzee (e), dog (g), human (h), mouse (m), nematode (n), opossum (o), rat (r), cow (w), thale cress (t), zebra fish (z) and vertebrate (V). The estimated count of predicted targets per miRNA per gene, or per miRNA only (for predictors do not allow inputting target gene), which is denoted by *, is given in the 'Number of targets' column; counts were estimated based on the corresponding papers or by testing the web servers. The possible formats of the input genes are by name, by sequence or by either name or sequence; 'none' denotes that searching particular genes is not allowed. 'new miRNA' shows whether a given method allows to predict new miRNAs.; methods that allow inputting miRNA sequences can be used to predict new miRNAs and are annotated with ✓, otherwise ✗. 'Impact/popularity' is assessed using the number of times a given method was highlighted and considered in the 15 review papers listed in Supplementary Table S2; 'Number citations' lists the average count of citations per year since published collected in September 2013 using ISI Web of Knowledge.

of species including human and mouse, and four of them also predict for rat or chicken. Four recent methods (HuMiTar [48], TargetMiner [49], MultiMiTar [70] and miRcode [72]) focus on human mRNAs, and TargetBoost [61] works only in worms.

Next, we analyze format of the inputs. The target genes can be specified by the name or identifier, by the mRNA sequence or are preloaded and the user is not allowed to enter them. Entering the name (e.g. GenBank Accession, NCBI gene ID and/or name) is arguably convenient but it also limits the prediction to the mRNAs that are available in the considered reference database(s). Allowing the user to provide mRNA sequence alleviates this drawback. Six predictors (MicroInspector [60], STarMir [66], PITA [67], MultiMiTar [70], miREE [71] and miRmap [41]) accept either the name or the sequence, while 3 and 11 programs accept only sequences or names, respectively. The miRNAs can be inputted in two formats: by name and/or by sequence. Again, although it may be convenient to specify miRNAs by their names, this is a rather substantial drawback, which does not allow predicting for novel miRNAs that are nowadays discovered at a rapid pace. Six methods that offer web servers (TargetScan [44], DIANA-microT [79], MicroInspector [60], PITA [67], miREE [71] and miRmap [41]) accept either the miRNAs name or the sequence, while 3 and 10 only take the sequences or the names, respectively. Table 3 reveals that 12 methods can predict targets of novel miRNAs.

When considering the outputs, the number of predicted targets varies widely between methods. Table 3 reports that while most methods predict a few targets per gene per miRNA, some predict hundreds, while miRanda [98] generates hundreds of thousands of targets per miRNA.

One way to measure impact/popularity of a given method is to analyze its inclusion in prior reviews. Considering the 16 reviews (Supplementary Table S2), 29 of the 38 methods were included in at least one review and 11 in five or more. Moreover, five reviews highlighted/recommended certain predictors. TargetScan [44] and TargetScanS [56] were recommended in three and four reviews, respectively; DIANA-microT [54] and RNAhybrid [47] twice, and EMBL method [62], PicTar [46], EIMMo [65] and PITA [66] once. We also calculated the average citation counts per year since a given predictors was proposed, using the Web of Knowledge. Table 3 reveals that 21 of the 38 methods receive on average >10 citations per year and all methods published before 2008 receive at least five citations per year. Three early methods receive >100 citations every year. TargetScan/TargetScanS [56] is on the extreme end (400+ citations per year), and this could be attributed to its popularity and convenient availability, the fact that empirical studies often compare to this predictor, and because it is widely used in practical applications.

Empirical evaluation of representative miRNA target predictors

We empirically evaluate seven representative target sequence-based predictors, i.e. methods that predict targets from miRNA and mRNA sequences, which are conveniently available to the end users that predict for human and mouse, and which provide sufficiently rich set of outputs. The selection criteria are discussed in the 'Materials and Methods' and Supplementary Table S6. They include older (PicTar 2005 [46]) and newer (TargetScan 6.2 [101], DIANA-microT-CDS [79], miRanda 2010 [40], EIMMo3 [65], mirTarget2 v4 [43] and miRmap v1.1 [41]) approaches that use a variety of types of predictive models. The predictions, which were collected using their web servers or precomputed predictions, consist of binding targets (mRNA sequences and/or positions of the binding site on mRNA) and the corresponding propensities (real-valued scores that quantify probability of the miRNA:target interaction). Table 4 and Supplementary Table S7 summarize results of the assessment at the gene level (to predict mRNAs that interact with a given miRNA) on the TEST_gene data set and the duplex levels (to predict whether a given fragment on mRNA interacts with a given miRNA) on the TEST_duplex data set. A given miRNA:target pair was predicted as functional if the target was predicted using the corresponding miRNA; the remaining targets were assumed to be predicted as nonfunctional and the corresponding propensity was set to 0. When assessing the gene level predictions, we scored a given gene using the sum of propensities among all its predicted target sites for a given miRNA. Because these seven methods were initially published before 2012, we use experimentally validated miRNA targets that were published after 2012 to perform the empirical assessment. This limits a bias caused by a potential overlap between our benchmark data and data used to develop a given method.

Considering the predictions of the miRNA:mRNA duplexes, TargetScan and DIANA-microT secure the highest AUC values of 0.674 and 0.673, respectively. Moreover, DIANA-microT has the highest MCC, which improves over the second best TargetScan by 0.073 [relative improvement of (0.273–0.200)/0.200*100% = 36.8%]. TargetScan offers the highest sensitivity, i.e. it correctly predicts the largest fraction of the functional duplexes. On the other hand, PicTar has the highest specificity, i.e. it correctly predicts the largest number of the nonfunctional duplexes. This means that functional targets predicted by PicTar are likely to be functional. DIANA-microT offers the highest SNR of correct to incorrect functional predictions (SNR+). TargetScan has the highest SNR− (SNR for the nonfunctional predictions), relatively good SNR+ and very good PNR (ratio of the number of predicted to native functional duplexes). PNR value of TargetScan reveals that it only slightly underpredicts, by 3.8%, the number of functional duplexes. The other methods, except for miRmap and EIMMo, underpredict the functional duplexes by a large margin. We illustrate relation between predictive quality (SNR values) and the outputted propensities binned to 10 intervals in Supplementary Figure S3A. The number of predicted duplexes and their SNR values in each interval are denoted by size and color of the bubbles (dark blue for accurate predictions), respectively. Alternating red and blue bubbles for a given predictor indicate that values of its propensity do not correlate with the underlying predictive quality. All methods have blue bubbles for propensity of 0, which means that they predict the nonfunctional duplexes well. However, predicted functional targets (propensity > 0) are often inaccurate (red bubbles) particularly for lower values of propensity. DIANA-microT predicts well when its propensity > 0.7, and miRmap and TargetScan when > 0.4 and 0.8, respectively. Analysis of statistical significance reveals that the differences in the AUC values (results above diagonal in Supplementary Table S7) are not statistically significant between TargetScan, DIANA-microT and miRmap. However, these three predictors are significantly better than the other four methods (P-value ≤ 0.001).

Table 5 analyzes anticipated predictive performance at the duplex level based on information that is available before the prediction is performed, including the nucleotide composition of the seed region and the overall size of the input miRNA sequences. The hints summarized in this Table could guide selection of a predictor based on the miRNA sequence. Most methods, especially TargetScan, DIANA-microT and miRmap,

Table 4. Comparison of predictive performance at the gene level (TEST_gene data set) and at the duplex level (TEST_duplex data set)

Prediction type	Predictor	AUC	MCC	Sen.	Spe.	Prec.	SNR+	SNR-	PNR
At the duplex level	TargetScan	**0.674**	0.200	**0.823**	0.389	0.855	1.346	**2.194**	**0.962**
	DIANA-microT	0.673	**0.273**	0.627	0.722	**0.908**	**2.256**	1.934	0.690
	miRmap	0.658	0.158	0.741	0.444	0.854	1.333	1.713	0.867
	miRanda	0.560	0.081	0.437	0.667	0.852	1.310	1.184	0.513
	EIMMo	0.552	0.116	0.696	0.444	0.846	1.253	1.463	0.823
	PicTar	0.538	0.069	0.272	**0.806**	0.860	1.400	1.107	0.316
	MirTarget2	0.519	0.055	0.285	0.778	0.849	1.282	1.088	0.335
At the gene level	TargetScan	**0.748**	0.386	0.733	0.652	0.733	2.108	2.446	**1.000**
	EIMMo	0.725	**0.391**	0.707	0.687	0.746	2.257	2.342	0.947
	miRmap	0.714	0.353	**0.800**	0.539	0.694	1.736	**2.696**	1.153
	DIANA-microT	0.637	0.225	0.520	0.704	0.696	1.759	1.467	0.747
	miRanda	0.636	0.239	0.467	0.765	0.722	1.988	1.435	0.647
	MirTarget2	0.627	0.298	0.327	**0.922**	**0.845**	**4.174**	1.369	0.387
	PicTar	0.588	0.196	0.340	0.835	0.729	2.058	1.265	0.467

We evaluate seven representative targets predictors. We measure area under the ROC curve (AUC), Matthews correlation coefficient (MCC), sensitivity (Sen.), specifity (Spe.), precision (Prec.), signal-to-noise ratio for predicted functional (SNR+) and predicted nonfunctional targets (SNR−) and predicted-to-native functional target ratio (PNR). Methods are sorted in the descending order by their AUC values. The best value of each measurement across all the predictors is given in bold font.

Table 5. Relation between predictive quality measured with AUC and compositional characteristics of the input miRNAs for predictions at the duplex level (TEST_duplex data set)

Predictor	Size of miRNAs			A count			C count			G count			U count		
	Short	Medium	Long	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
TargetScan	+	++	−	+	++	+	++	=	++	+	++	+	+	+	+
DIANA-microT	−	++	=	++	++	−	++	=	++	+	++	−	+	+	++
miRmap	=	++	−	++	++	=	++	−	++	+	++	+	+	+	+
miRanda	=	+	−	+	=	−	=	−	+	=	−	=	=	=	−
EIMMo	=	+	−	−	+	−	+	−	=	=	=	=	−	+	−
PicTar	−	=	−	−	−	=	=	−	=	−	−	+	=	=	−
MirTarget2	−	=	−	=	−	−	=	−	=	−	−	=	−	−	−

The compositional characteristics include the size of miRNA and the count of each nucleotide type in the seed region. The sizes are divided into short (<22 nt), medium (=22 nt) and long (>22 nt). The count of nucleotides in the seeds of miRNAs is grouped into low (<2 nt), medium (=2 nt) and high (>2 nt). The AUC values obtained by a given predictor are coded as: '−' for [0, 0.55], '=' for (0.55, 0.6], '+' for (0.6, 0.7] and '++' for (0.7, 1.0].

predict well for medium-sized (22 nucleotides long) miRNAs. The predictions for longer miRNAs are generally less accurate. Considering the nucleotide content in the seed region, the same three methods provide high-quality predictions for miRNAs when the seeds have 2 adenines or 2 guanines, and <2 cytosines. DIANA-microT also predict well for <2 adenines and >2 uracil and miRmap for <2 adenines. Overall, we recommend TargetScan, DIANA-microT and miRmap because their AUCs >0.7 for specific types of miRNAs.

The overall prediction quality is higher and ranking of the methods is slightly different for the predictions on TEST_gene data set when compared with the TEST_duplex data set (Table 4). TargetScan secures the highest AUC, while EIMMo moves up to the second place and provides the highest MCC. TargetScan improves in AUC over the second best EIMMo by 0.023 (relative improvement of 3.2%) and over miRmap by 0.043 (relative improvement of 4.8%). miRmap offers the highest sensitivity and TargetScan provides arguably the best balance between sensitivity and specificity (both scores are high and similar). MirTarget2 is the most conservative method given its highest specificity, precision and SNR+, i.e. it predicts only a few functional targets but with high success rate. The PNR values reveal that TargetScan predicts exactly the right number of functional genes and EIMMo only 5.3% too few. Supplementary Figure S3B shows relation between predictive quality (SNR values) and the propensities generated by the prediction methods. Interestingly, predictions associated with higher propensities are more likely to be more accurate, as evidenced by the presence of (dark) blue bubbles. As a highlight, EIMMo predicts well in every propensity bin, and the targets predicted by TargetScan and miRanda with propensities >0.3 and 0.4, respectively, are characterized by high SNR values. Analysis of statistical significance of differences in the AUC values (results below diagonal in Supplementary Table S7) reveals that TargetScan's results are significant better (P-value ≤ 0.001) compared with the other predictors. AUCs of EIMMo and miRmap are not significantly different and significantly higher than AUCs of the other four methods (P-value ≤ 0.001). We also analyze relation between predictive performance at the gene level and the number of target sites predicted in a given gene (Supplementary Figure S3C). Most methods, except for MirTarget2 and miRanda, can predict three or more target sites per gene for a given miRNA. We observe that predictive quality for genes for which at least two sites are predicted is better (bubbles have darker blue color), particularly for EIMMo, TargetScan and miRanda. This suggests that for these

predictors higher number of predicted sites could be used as a marker of higher predictive quality.

Predictions at the transcriptome/proteome scale on the TEST_geo and TEST_psilac data sets are evaluated at different thresholds that define the fraction of the most repressed and most overexpressed genes that are annotated as functional and nonfunctional, respectively (Figure 1). AUCs are generally higher at the gene level (TEST_geo data set) than at the protein level (TEST_psilac data set). Considering the three gene-level data sets, the ranking of the methods on the TEST_psilac data set is the same as on the TEST_gene data set, and slightly different on the TEST_geo data set. Based on the microarray data, miRmap achieves the best AUC, which is comparable with the AUC of TargetScan and EIMMo. These three predictors have AUCs >0.7 when evaluated on the top 4% of genes with largest expression changes; using this threshold, on average each miRNA targets 176 mRNAs. We note miRmap was originally trained and tested on two of the three microarrays from the TEST_geo data set, so its predictive quality on this data set could be overestimated. Considering the pSILAC data, only TargetScan provides AUC >0.7 when using top 1% of proteins for which expression levels change most; this threshold results in an annotation where on average each miRNA regulates 39 proteins. Overall, the AUC values decrease when more ambiguous genes (genes for which expression changes are weaker) are included, i.e. the fraction of the included repressed and overexpressed genes is higher. Analysis of the MCC values (Supplementary Figure S4A and B) leads to similar conclusions. TargetScan, EIMMo and miRmap secure the highest values of this index.

We also calculate the average logarithm of the fold change of the top predicted genes (i.e. genes that obtain the highest propensity score) for each method to assess whether higher propensity implies better predictive performance (Supplementary Figure S4E and F). Genes with high propensity of binding predicted by MirTarget2 are characterized by large expression changes, with almost 3-fold change for the top 10 targets predicted for each miRNA. This strong result is consistent with high precision at the gene level on the TEXT_gene data set, which is secured by this method. High values of propensities generated by TargetScan are also indicative of higher changes in the gene expression levels, while the results of the other methods are inconsistent between the two data sets. We note that expression level changes are larger on the TEST_psilac data set, which is probably due to a different amount of mRNAs and available miRNA in the cell [102] and differences in the experimental conditions. This also hints that it would be

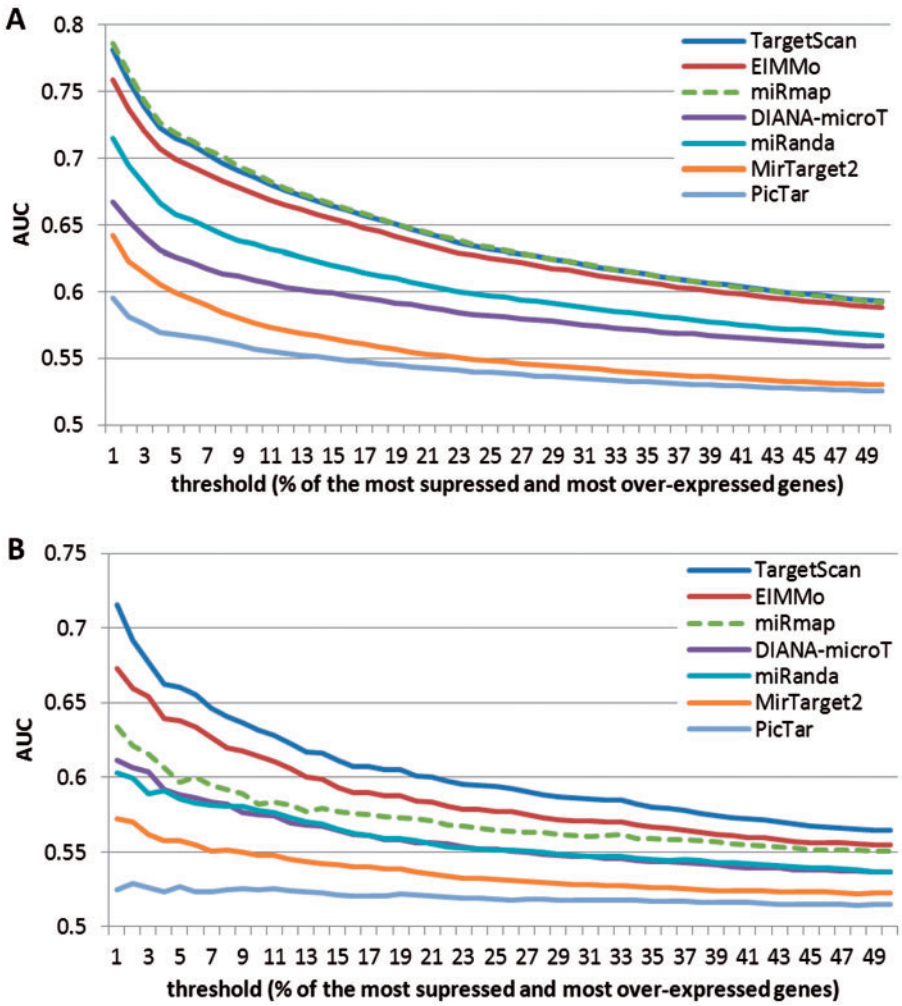

Figure 1. Relation between AUC values and the threshold used to define the functional (most suppressed) and nonfunctional (most overexpressed] genes for the predictions on the TEST_geo (panel A] and TEST_psilac (panel B] data sets. Methods are sorted in the same order with those on TEST_gene data set. miRmap that was trained on the gene expression data is given with the dashed line. A colour version of this figure is available online at BIB online: http://bib.oxfordjournals.org.

implausible to predict absolute gene expression changes solely based on the miRNA and mRNA sequences. From the PNR curves (Supplementary Figure S4C and D), we observe that all methods, except for miRmap on the TEST_geo data set, underpredict functional targets by a substantial margin. Considering that these data sets may miss native functional genes that are associated with smaller expression level changes and that some of the targets genes could be annotated based on an indirect interaction with the miRNAs, the list of functional targets defined solely by the expression changes could be incomplete and may include FP. Therefore, we do not expect PNR values close to 1 on the TEST_geo and TEST_psilac data sets.

Discussion

We reviewed 38 miRNA target predictors from all significant perspectives including their prediction models, availability, impact, user friendliness and protocols and measures that are used to evaluate their predictive performance. We found that standardized evaluation procedures are urgently needed because currently predictors are evaluated using different measures, different test protocols and using vastly different data sets. This hinders comparison among these methods and appropriate selection by the end users. To this end, we empirically and systematically compared seven representative predictors on four benchmark data sets, considering prediction of miRNA:mRNA duplexes and targets genes and proteins.

We found that although certain methods, like TargetScan and miRmap, offer high overall predictive quality, there is no universally best predictor. For instance, PicTar and MirTarget2 provide predictions with high specificity and low number of FP (incorrectly predicted functional genes/duplexes). Thus, these two methods are suitable for users that would like to obtain a small subset of accurately predicted functional duplexes or genes. EIMMo predicts well at the gene level. We observe that the count of functional target sites or genes predicted by TargetScan is the closest to the native count (PNR value close to 1), and thus, this method should be used to accurately estimate the number of miRNA targets. We found that genes predicted as functional based on a higher number of sites are more likely to be accurate, particularly for the EIMMo and TargetScan predictors. Finally, the benchmark data sets and empirical results that we provide are useful to develop and comparatively assess future prediction methods.

We observe that predictions at the duplex level are characterized by lower predictive quality than the predictions of targets genes. This agrees with intuition that predicting target sites should be more difficult than predicting target genes that offer more input information (longer mRNA sequence). Moreover, our estimates of the predictive performance are often lower than the estimates from the original publications. Possible reasons are as follows: (i) we use experimental validated data, which is likely more challenging than the artificial data that were used to assess previous predictors; (ii) the nonfunctional validated duplexes that we use have relatively many Watson–Crick (WC) base pairs in the seed regions (83% have at least six pairs, see Supplementary Table S8). These sites were likely hypothesized to be functional, refuted and thus annotated as nonfunctional. This is why they have such seeds, which in turn makes them more challenging to separate from the functional duplexes when compared with a more 'random' site; and (iii) miRanda, PicTar, EIMMo and MirTarget2 provide only precomputed predictions, which may not include most up-to-date miRNA and transcript databases. Unfortunately, we could not compare results with the previous reviews [24, 25, 103] because they did not consider a balanced selection of measurements (e.g. only provided sensitivity and precision, which ignore TN), and such one-sided evaluation would not be meaningful.

Our review offers in-depth insights that could be used by the end users to select prediction methods based on their predictive performance (Table 4) and their input miRNAs (Table 5). We also provide several practical observations that consider specifics of applications of interest. Arguably, the commonly considered characteristics of the applications of the miRNA target predictors include the need to consider novel miRNAs and to focus on certain regions in the mRNA, to predict a more complete or smaller and more accurate list of targets, to predict for a large set of miRNAs, to tweak desired parameters of the miRNA–mRNA interaction and to generate propensities for the predicted interactions. We address these characteristics as follows:

- Only some methods can predict targets for novel miRNAs (see 'New miRNA' column in Table 3).
- Applications that focus on particular regions (e.g. 5′UTR, CDS, promoters) should use predictors that were designed to consider these regions (see 'target region' column in Table 3).
- Some methods generate few and potentially more accurate targets, while some predict a larger and more complete set of targets that may include more FP (see 'Number of targets' column in Table 3). Users should choose an appropriate method depending on whether they look for a more complete or a more accurate set of targets.
- When predicting for a large number of miRNAs, the downloadable precomputed results or methods that provide APIs should be used (see 'batch search' in the 'Note' column in the Supplementary Table S6).
- The end users should apply predictors with tunable seed type parameter, such as PITA, when searching for targets that use a particular seed type. Also, when aiming to find targets with low number of WC pairs in the seed region, only some predictors that consider such targets, like miREE, can be used.
- When predicting the target site, the methods that can only predict target genes cannot be used (see 'Target site tracking' column in Supplementary Table S6).
- Only some predictors provide predictions with the associated propensities of the interaction; many methods only provide binary (functional versus nonfunctional) predictions (see 'Score' column in Supplementary Table S6)

Although undoubtedly computational miRNA target predictors are useful and their predictive performance is relatively good, we suggest several areas where further improvements are possible:

- Current methods use many different predictive models. In contrast to other areas of bioinformatics, the empirical (knowledge-based) models do not outperform the heuristic models. This could be due to the low quantity of training data, use of artificial training data (randomly generated nonfunctional targets) and unbalanced nature of the data (low number of nonfunctional targets). Thus, one of the future aims should be to improve the quality and quantity of the training data.
- Further improvements in predictive quality could be attained by finding and using not yet known characteristics of miRNA:target interactions. For instance, recently cis-element was used to connect primary miRNAs to their potential targets [75], and Gene Ontology annotations and protein–protein interaction networks

were used to filter target predictions [104]. Also, the CLIP data has been used to annotate functional targeting sites; however, not much effort so far was made to use these data as a filter to improve specificity of the current prediction methods [105].

- We emphasize the need to introduce and maintain higher standards in evaluation of predictive performance, as this would provide a cleared picture of current state of this field. Similar to our empirical study (see 'Materials and Methods'), this should include a comprehensive set of measurements, statistical tests and use of independent (from the training data) benchmark data sets.
- Lastly, the outputs generated by the predictors should be expanded to provide more value for the end users. Some of the possible suggestions include providing location of predicted target sites, allowing predicting targets of novel miRNAs and predicting the strength of the binding with the help of the gene expression data [106].

Supplementary data

Supplementary data are available online at http://bib.oxfordjournals.org/.

Key Points

- Computational miRNA target prediction from sequence is essential to characterize miRNA functions and to develop miRNA-based therapeutics.
- We comprehensively summarize 38 miRNA target predictors and empirically evaluate seven methods on four benchmark data sets that annotate targets at the binding site, gene and protein levels.
- Current miRNA target prediction methods substantially vary in their predictive methodology, usability and predictive performance.
- We offer insights for the end users to select appropriate methods according to their specific application and we discuss advantages and disadvantages of the considered predictors.
- New miRNA target predictors are needed, particularly focusing on the high-throughput predictions, improved predictive performance and provision of an expanded range of predicted outputs.

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