Analysis of microRNA precursors in multiple
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Ivani de Oliveira Negrão Lopes

SERVIÇO DE PÓS-GRADUAÇÃO DO ICMC-USP

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***Advisor:* Prof. Dr. André Carlos Ponce de Leon Ferreira de Carvalho**

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***Orientador:* Prof. Dr. André Carlos Ponce de Leon Ferreira de Carvalho**

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Abbreviations

- **bp**: base-pair
- **CDS**: coding DNA Sequence or Coding sequence
- **DNA**: deoxyribonucleic acid
- **FS**: feature set
- **MFE**: minimum free energy of folding
- **miRNA**: microRNA
- **mRNA**: messenger RNA
- **ncRNA**: non-coding RNA
- **nt**: nucleotides
- **OOB**: out-of-bag error
- **pdf**: probability density functions
- **pre-miRNA**: microRNA precursor
- **RFI**: relative feature importance
- **RNA**: ribonucleic acid
- **RNA-seq**: RNA sequencing
- **sRNA**: small RNA
- **Ss**: subset of feature set
- **stRNA**: small temporal RNA (also microRNA)
- **tmRNA**: target mRNA
- **UTR**: Untranslated region
- **3p**: 3' or 3 prime end of a genomic sequence
- **5p**: 5' or 5 prime end of a genomic sequence

Abstract

RNA Sequencing has recently emerged as a breakthrough technology for microRNA (miRNA) discovery. This technology has allowed the discovery of thousands of miRNAs in a large number of species. However, despite the benefits of this technology, it also carries its own limitations, including the need for sequencing read libraries and of the genome. Differently, *ab initio* computational methods need only the genome as input to search for genonic locus likely to give rise to novel miRNAs. In the core of most of these methods, there are predictive models induced by using data mining techniques able to distinguish between real (positive) and pseudo (negative) miRNA precursors (pre-miRNA). Nevertheless, the applicability of current literature *ab initio* methods have been compromised by high false detection rates and/or by other computational difficulties. In this work, we investigated how the main aspects involved in the induction of predictive models for pre-miRNA affect the predictive performance. Particularly, we evaluate the discriminant power of feature sets proposed in the literature, whose computational costs and composition vary widely. The computational experiments were carried out using sequence data from 45 species, which covered species from eight phyla.

The predictive performance of the classification models induced using large training set sizes ($\geq 1,608$) composed of instances extracted from real and pseudo human pre-miRNA sequences did not differ significantly among the feature sets that lead to the maximal accuracies. Moreover, the differences in the predictive performances obtained by these models, due to the learning algorithms, were neglectable. Inspired by these results, we obtained a feature set which can be computed 34 times faster than the less costly among those feature sets, producing the maximal accuracies, albeit the proposed feature set has achieved accuracy within 0.1% of the maximal accuracies. When classification models using the elements previously discussed were induced using small training sets (120) from 45 species, we showed that the feature sets that produced the highest accuracies in the classification of human sequences were also more likely to produce higher accuracies for other species. Nevertheless, we showed that the learning complexity of pre-miRNAs vary strongly among species, even among those from the same phylum. These results showed that the existence of specie specific features indicated in previous studies may be correlated with the learning complexity. As a consequence, the predictive accuracies of models induced with different species and same features and instances spaces vary largely. In our results, we show that the use of training examples from species phylogenetically more complex may increase the predictive performances for less complex species. Finally, by using ensembles of computationally less costly feature sets, we showed alternative ways to increase the predictive performance for many species while keeping the computational costs of the analysis lower than those using the feature sets from the literature.

Since in miRNA discovery the number of putative miRNA loci is in the order of millions, the analysis of putative miRNAs using a computationally expensive feature set and or inaccurate models would be wasteful or even unfeasible for large genomes. In this work, we explore most of the learning aspects implemented in current ab initio pre-miRNA prediction tools, which may lead to the development of new efficient ab initio pre-miRNA discovery tools.

Resumo

O sequenciamento de pequenos RNAs surgiu recentemente como uma tecnologia inovadora na descoberta de microRNAs (miRNA). Essa tecnologia tem facilitado a descoberta de milhares de miRNAs em um grande número de espécies. No entanto, apesar dos benefícios dessa tecnologia, ela apresenta desafios, como a necessidade de construir uma biblioteca de pequenos RNAs, além do genoma. Diferentemente, métodos computacionais ab initio buscam diretamente no genoma regiões prováveis de conter miRNAs. A maioria desses métodos usam modelos preditivos capazes de distinguir entre os verdadeiros (positivos) e pseudo precursores de miRNA - pre-miRNA - (negativos), os quais são induzidos utilizando técnicas de mineração de dados. No entanto, a aplicabilidade de métodos ab initio da literatura atual é limitada pelas altas taxas de falsos positivos e/ou por outras dificuldades computacionais, como o elevado tempo necessário para calcular um conjunto de atributos. Neste trabalho, investigamos como os principais aspectos envolvidos na indução de modelos preditivos de pre-miRNA afetam o desempenho preditivo. Particularmente, avaliamos a capacidade discriminatória de conjuntos de atributos propostos na literatura, cujos custos computacionais e a composição variam amplamente. Os experimentos computacionais foram realizados utilizando dados de sequências positivas e negativas de 45 espécies, cobrindo espécies de oito filos.

Os resultados mostraram que o desempenho preditivo de classificadores induzidos utilizando conjuntos de treinamento com 1608 ou mais vetores de atributos calculados de sequências humanas não diferiram significativamente, entre os conjuntos de atributos que produziram as maiores acurácias. Além disso, as diferenças entre os desempenhos preditivos de classificadores induzidos por diferentes algoritmos de aprendizado, utilizando um mesmo conjunto de atributos, foram pequenas ou não significantes. Esses resultados inspiraram a obtenção de um conjunto de atributos menor e que pode ser calculado até 34 vezes mais rapidamente do que o conjunto de atributos menos custoso produzindo máxima acurácia, embora a acurácia produzida pelo conjunto proposto não difere em mais de 0.1% das acurácias máximas. Quando esses experimentos foram executados utilizando vetores de atributos calculados de sequências de outras 44 espécies, os resultados mostraram que os conjuntos de atributos que produziram modelos com as maiores acurácias utilizando vetores calculados de sequências humanas também produziram as maiores acurácias quando pequenos conjuntos de treinamento (120) calculados de exemplos de outras espécies foram utilizadas. No entanto, a análise destes modelos mostrou que a complexidade de aprendizado varia amplamente entre as espécies, mesmo entre aquelas pertencentes a um mesmo filo. Esses resultados mostram que a existência de características específicas em pre-miRNAs de certas espécies sugerida em estudos anteriores pode estar correlacionada com a complexidade de aprendizado. Consequentemente, a acurácia de modelos induzidos utilizando um mesmo conjunto de atributos e um mesmo algoritmo de aprendizado varia amplamente entre as espécies.

Os resultados também mostraram que o uso de exemplos de espécies filogeneticamente mais complexas pode aumentar o desempenho preditivo de espécies menos complexas. Por último, experimentos computacionais utilizando técnicas de *ensemble* mostraram estratégias alternativas para o desenvolvimento de novos modelos para predição de pre-miRNA com maior probabilidade de obter maior desempenho preditivo do que estratégias atuais, embora o custo computacional dos atributos seja inferior.

Uma vez que a descoberta de miRNAs envolve a análise de milhares de regiões genômicas, a aplicação prática de modelos preditivos de baixa acurácia e/ou que dependem de atributos computacionalmente custosos pode ser inviável em análises de grandes genomas. Neste trabalho, apresentamos e discutimos os resultados de experimentos computacionais investigando o potencial de diversas estratégias utilizadas na indução de modelos preditivos para predição ab initio de pre-miRNAs, que podem levar ao desenvolvimento de ferramentas ab initio de maior aplicabilidade prática.

Introduction

RNA Sequencing (RNA-Seq) has recently emerged as a breakthrough technology for microRNA (miRNA) discovery. This technology has allowed the discovery of thousands of miRNAs in a large number of species. However, despite the benefits of this technology, it also carries its own limitations, including the need for the development of RNA-Seq libraries, the genome availability, posterior computational analysis and eventual experimental verification. Differently, *ab initio* computational methods use only the genome as input to search for genomic locus likely to give rise to novel miRNAs. As *ab initio* methods require only a genomic sequence as input, the main tasks in miRNA discovery using *ab initio* methods are the prediction of good miRNA candidates and posterior experimental validation. However, low sensitivity and/or low specificity of existing *ab initio* methods was reported in [24], whose methods identities were not mentioned. In another comparison, [66] showed that the specificity of microPred, a benchmark *ab initio* method in pre-miRNA prediction, achieved specificity of approximately 70%. Predictive methods with such low specificity can make the experimental validation of the predicted instances unfeasible, considering the large number of pre-miRNAs candidates. Additionally, most *ab initio* methods use quantitative features computationally expensive to extract, which can make their application in large genomes unfeasible.

Since most *ab initio* methods have been developed using data mining techniques, the understanding of how these techniques affect the predictive performance of these methods is the first step towards the development of more effective tools. For example, the choice of a learning algorithm, or a feature spaces or a set of training instances can insert learning biases that reduce the predictive performance of unseen instances. Current literature methods have reported high

and close predictive performances, even though they have been developed with different aims and being based on different data mining techniques.

In this work we investigated how the elements used to design classifiers to predict genomic locus likely to give rise to novel miRNAs can affect their classification performances. In the following sections, we provide a general biological background and describe the miRNA discovery problem according to the main computational approaches. The motivation, the general goals and the specific goals of this work are also described in this chapter.

1.1 Historical context

The central dogma of molecular biology states that biological systems are stored in hereditary units of DNA known as genes, which are copied into RNAs, which are translated into proteins [19, 61]. The latter fulfill not only most structural and catalytic aspects but also most regulatory functions, in all cells, from microbes to mammals [70]. As a consequence, it has been believed that the primary function of genes was to serve as protein templates. However, even though the central dogma framework remains true, the growing knowledge on the role of some non-protein-coding RNA genes (ncRNAs) has unraveled many other layers of transfer information. In fact, some ncRNAs have been known for a long time and have well-established functions, whereas others are in the process of functional characterization (reviewed in [71]).

Until recently, the expression level of protein coding genes was credited to transcription factors (TFs), which are proteins that either activate or repress the transcription of other genes [15]. However, new mechanisms of pos-transcription gene regulation have been unveiled since the discovery by [3] of a small temporal RNA (stRNA), called miRNA. The excitement towards the understanding of the participation of miRNAs in the gene regulation networks is still growing [4]. In addition to the important associations found between miRNA and development and cell differentiation [53], immunity [86], tumorigenesis [11, 17] and plant development [100], evolutionary analyses have shown evidences that the amount of miRNAs is highly correlated with morphological complexity [20, 33, 45, 68, 69, 71]. Because of the important role of miRNAs in pos-transcription gene regulation, the mechanisms involving the rising of novel miRNAs and the functional characterization of known miRNAs became active research fields in molecular biology in the recent years.

1.2 What is miRNA?

MiRNA is a small ncRNA gene of approximately 17-25 nucleotides (nt) that modulates pos-transcription the stability of messenger RNA targets(tmRNA) and their rate of translation into proteins [44]. The mechanism of gene regulation by miRNAs was first observed through the characterization of two genetic loci involved in the control of developmental timing in

Caenorhabditis elegans (*C. elegans*): *lin-4* and *lin-14* [4, 52]. Through genetic analysis, *lin-4* has been previously known to repress the translation of *lin-14*.

The novelty came with the realization that *lin-4* was a ncRNA processed from a short hairpin precursor and that the repression of *lin-14* was induced by partial sequence complementarity between the *lin-4* RNA and the *lin-14* 3p UTR. Subsequently, the findings that *lin-4* not only regulates *lin-14* but also *lin-28* [75], suggested that the *lin-4-lin-14* way of gene-regulatory interaction could be a more general phenomenon. Actually, the regulation of *lin-14* and *lin-28* by *lin-4* showed that distinct genes could acquire functional *lin-4* sites, indicating a potential evolutionary flexibility for this type of antisense interaction [4]. Currently, miRNAs have been found in species from almost all kingdoms.

1.3 miRNA biogenesis

MiRNA biogenesis is a complex process that requires the participation of several enzymes. There are many peculiarities in the biogenesis, depending on the kingdom of the species and the origin of the miRNA. The descriptions presented here are based on [100] and [5]. First, a miRNA gene is transcribed to a primary miRNA (pri-miRNA) through Polymerase II (Pol II) enzymes, which is usually a long sequence of more than several hundred nucleotides. A characteristic feature of the pri-miRNA is the fold-back, or hairpin-like shape.

In the second step, the pri-miRNA is cleaved by to a stem-loop intermediate, called pre-miRNA. The cleavage is controlled by the Drosha RNase III endonuclease in animals (Figure 1.1 A) or by Dicer-like 1 enzyme (DCL1) in plants (Figure 1.1 B). Third, in animals, pre-miRNAs are transported by Exportin 5 from the nucleus into the cytoplasm followed by formation of miRNA:miRNA* duplex and mature miRNAs by another RNase III-like enzyme called Dicer. Differently from animals, plant miRNAs are cleaved into miRNA:miRNA* duplex by DCL1 in the nucleus, rather than in the cytoplasm. Afterwards, the duplex is translocated into the cytoplasm by HASTY, the plant orthologus of Exportin 5.

In the cytoplasm, both plant and animal miRNAs are unwound into single strand mature miRNAs by helicase. The star strand and the loop usually degrades and the mature miRNA enters in a ribonucleoprotein complex, known as the RNA-induced silencing complex (RISC) where they regulate tmRNA. As Figure 1.1 shows, two kinds of miRNA:tmRNA pairing can occur in animals (Figure 1.1 A): near-perfect pairing, leading to the degradation of the tmRNA and partial pairing which inhibits the translation of the tmRNA. In plants (Figure 1.1 B), the pairing is near-perfect, causing the tmRNA degradation.

Because the exact position of the Drosha and DCL1 signatures can rarely be identified, the indication of these sites is not a requirement for the deposit of a pre-miRNA in miRBase [29], the largest repository of experimentally validated miRNA. Concomitantly, the nomenclature

Figure 1.1: Biogenesis of canonical animal (A) and plant (B) miRNAs.

pre-miRNA used in this text may also refer to the extended hairpin, the stem-loop hairpin or the even pri-miRNA.

1.4 Computational prediction of miRNAs

Computational methods are important tools in miRNA discovery. They have been used in many different ways, but always aiming to reduce the amount of time and effort between the detection of putative miRNA loci and the experimental verification *in vivo*. As miRNAs are short genes, and consequently their short sequences are uninformative, computational detection of miRNAs from genomic sequences initially searches for sequences resembling known pre-miRNAs. The first batch of computational tools for miRNA discovery used comparative genomic approaches. Because many miRNAs are conserved in close species, these algorithms restricted the search space to conserved genomic regions obtained through multiple sequence alignment of phylogenetically close species [39, 48, 58, 77, 88]. Those approaches have the advantages of reducing the search space and the false detection rates.

Due to the discovery of species-specific [80] and non-conserved [59] miRNAs, in addition to recent studies which have indicated that miRNA evolution seems to progress at a high rate and that conserved miRNAs may represent only a small fraction [2, 56, 63], comparative genomic approaches have been put aside. The next generation of computational tools for pre-miRNA

prediction was called *ab initio*, because no phylogenetic conservation was required [85]. In these approaches, putative pre-miRNAs are searched directly in the investigated genomic sequence without additional information on the conservation of specific genomic loci. To reduce the search space, some of these tools relied on the fact that many miRNAs occur in clusters in the genome to predict miRNAs neighboring other known miRNAs [66, 76, 85]. In this case, both the genome and known miRNA are used as input. Other tools such as MiRank [93] and miRNAFold [87] are “totally” *ab initio*, in the sense that the genome is the only input. Currently, miRNA discovery from RNA-Seq libraries became the *de facto* method. In the following subsections, we introduce the fundamentals and elements of miRNA prediction through *ab initio* genomic screening and through RNA-Seq data.

1.4.1 *Ab initio* genomic screening

MiRNA discovery using *ab initio* genomic screening evaluates sliding windows of length L throughout the genome. Since there is no parameter in the sequence level that gives any clue of what would be a plausible putative locus, the initial evaluation of the window is made at the structure level (Figure 1.2). The secondary structure represents the sequence intramolecular accommodation demanding the lowest free energy of folding (MFE), which can be predicted by an RNA folding software, such as RNAfold [62] and Mfold [101]. However, true and pseudo hairpins may have similar structural features, as illustrated in Figure 1.3 A. This figure shows the structural similarities between the predicted secondary structure of the *Bombyx mori* pre-miRNA bmo-mir-2855 and a pseudo hairpin taken from *Bombyx mori* protein coding sequences (cds) [14]. Both predicted structures have a typical hairpin with one loop, a close number of bulges and dangling ends. Likewise, Figure 1.3 B shows visual similarities between the predicted secondary structures of the *Picea abies* pre-miRNA pab-MIR3711 and a pseudo hairpin taken from *Denio rerio* cds [14]. True and pseudo hairpins have three loops, close number of bulges and dangling ends. This simple illustration shows that the distinction between true and pseudo hairpins also depends on intrinsic parameters. Moreover, pre-miRNA secondary structure varies largely from the typical hairpin-loop to highly complex structures, which is more prevalent in plants.

Since the genome content of many Eukaryotes is in the order of a billion nucleotides, the major challenge of genomic screening approaches is to discard implausible windows using inexpensive computational criteria. Additionally, the window size is usually fixed, which may not be the most efficient way to select putative hairpins, since the length distribution of pre-miRNAs varies substantially.

1.4.2 RNA-Seq

RNA-Seq, also called deep sequencing of transcriptome, can reveal the identities of most RNA species inside a cell, providing tens to hundreds of millions of sequence “reads” [18]. These reads

only small reads (approx. 25 nt long) are sequenced [18]. The computational challenge consists in distinguishing miRNAs from other small RNA (sRNA) types and degradation products [24].

Computational tools able to detect new miRNAs from an RNA-Seq library depend on the reference genome. Through a sequence alignment tool, such as Bowtie 2 [49], each unique read is mapped to the reference genome and the genomic locus surrounding the read is analyzed for consistency with miRNA biogenesis. This rationale assumes that the loop, the miRNA and the miRNA* strands were sequenced and their mapping pattern allows the miRNA:miRNA* 2 nt overhang at the 3p ends (Figure 1.4 A). Usually, the mature strand is the most frequent read followed by the star strand and the loop. However, sometimes, the star strand has similar frequency distribution to the mature strand, other times the star strand and/or the loop may not even be sequenced. The last situation can occur if the miRNA is lowly expressed in the sequenced genetic material.

Figure 1.4: Identification of miRNAs using libraries of sRNA sequences. RNA reads representing miRNA:miRNA* duplex form a 2nt 3p overhang (A), but other patterns can occur, such as the observed in transitional miRNAs (B). The distribution of non miRNA reads throughout the putative hairpin is random (C). Figure adapted from [8]

When the sRNA reads are aligned in accordance with the biogenesis model and their frequency distributions are consistent with the expected pattern, the read with the highest frequency is more likely to be the mature miRNA. However, because pseudo hairpins are highly frequent in complex genomes, such as human [7], additional analyses of the putative miRNA locus based on other characteristic features, such as the energy stability, are performed to reduce false detection. Moreover, as described in [8], hairpin loci that are in the process of evolving towards a miRNA function, called transitional miRNAs, present more variation in their read distribution over the hairpin (Figure 1.4 B). In these situations, subsequent analyses using characteristic features from real pre-miRNAs are fundamental to indicate if the putative miRNA

is worth of additional experimental evidence for miRNA biogenesis [8]. Lastly, reads unlikely to be miRNAs are usually mapped to the putative hairpin randomly (Figure 1.4 C). Disadvantages of miRNA discovery from RNA-Seq libraries are the need for read library preparation and the eventual loss of information due to the low or non-expression of certain miRNAs in the genetic material used to create the reads library.

1.4.3 miRNA abundance

The number of miRNAs in genomes is still unclear, even in the human genome, which has currently the largest amount of known miRNAs. Estimates of the number of human miRNAs by [7] in 2005 were surpassed five years latter by the number of miRNAs deposited in miRBase release 15 [46]. Recent estimates raised evidences that approximately 60% of all human genes are regulated by miRNAs [25], distinctively to plants, whose estimate is approximately 1%. Although these numbers may not be the best indicator for the amount of miRNA, they reflect the importance of this large class of regulatory genes.

1.5 Analysis of putative pre-miRNA

In the previous section, we mentioned that computational discovery of miRNAs depends on analyses of genomic loci called putative pre-miRNAs. These analyses use intrinsic and extrinsic features computed from true (+) and pseudo/background (−) pre-miRNA sequences. Each sequence is represented by a feature vector composed of features computed from the respective sequence. Hundreds of features have been used to evaluate putative pre-miRNAs. As mentioned in [2], these features are usually placed into four categories:

- sequence;
- sequence and/or structure;
- thermodynamic;
- probabilistic properties.

The definition of these features are presented in Section 2.1, Chapter 2.

Feature vectors computed from positive and negative sequences are later used to induce models to predict pre-miRNAs. Classification algorithms are the most common approach. Among them we can mention, support vector machines (SVMs)[6, 27, 34, 78, 94, 95, 97, 98], random forest (RF) [30, 42], the relaxed variable kernel density estimator (RVKDE) [13] and a generalized Gaussian components based density estimation (G²DE) [37]. Probabilistic models, like Naïve Bayes, were used to score putative miRNAs [99] and pre-miRNA [24, 55]. However, it has been suggested that this learning algorithm does not have a major effect in the predictive accuracy [2]. Moreover, once

a classification model has been obtained, the computational cost of the analysis ends up being the computational cost of computing the feature vector representing each sequence to be evaluated. Therefore, since the induction is a one time task whereas the evaluation of putative pre-miRNA is performed on millions of sequences, the training time is irrelevant compared to the feature's computational time. As pointed out in the previous paragraph, hundreds of features have been used in different sets. However, the predictive power of these features has not been analyzed in depth [2]. Moreover, some of these features may be redundant or even irrelevant for the problem. Additionally, these features vary substantially in computational complexity.

1.6 Motivation

Since millions of putative pre-miRNAs can be generated from a genome such as human [7], computational evaluation needs to take into consideration the computational cost of computing the features, in addition to the predictive accuracy of the induced models. Feature sets proposed in literature papers were obtained in order to maximize classification accuracy, driven by automatic feature selection methods or by evidences of their discriminant power. However, the computational cost of features is a critical factor if they are to be computed from millions of candidates. Moreover, it is still unclear if there is a feature set in the literature more likely to produce classifiers with higher predictive performance. Another important point to consider is the absence of information on the generalization of these feature sets throughout species within and between kingdoms. Finally, the following questions need to be answered: is it possible to lower the computational cost of the computational evaluation of putative pre-miRNAs without compromising the predictive performance of the classifier?

1.7 Objectives

Even though research for determining the role of miRNAs in diverse biological organisms has advanced significantly, what constitutes a miRNA is still not completely elucidated, which makes computational detection of miRNA a challenging task. The major challenge is to discard implausible candidates and retain candidates likely to be a miRNA locus using low computational cost criteria. Keeping these points in mind, our general objective is:

Provide guidelines for computational analyses of putative miRNA loci with low computational cost predictive models.

To accomplish the general objective, we focused on the following specific objectives:

1. To assess the discriminant power of feature sets frequently used in six tools proposed in the literature;
2. To assess the impact of learning algorithms in the predictive accuracy of classifiers;

3. To provide information on the generalization of the discriminant power of feature sets from the literature on animal and plant species;
4. To assess the worth of cross-species classification models;
5. To provide alternative feature sets able to reduce the time of computational analyses of putative miRNA.

1.8 Proposals

1. **Identification of discriminant features:** The predictive performance of tools found in the literature are very close, even though they use different feature sets. However, a deep comparison should be performed under the same learning algorithm, training and test sets. These investigations were conducted in the scope of human pre-miRNA, which is currently the largest set of positive examples. We compared seven feature sets from the literature, named here as FSi , for $i = 1, \dots, 7$.
2. **Effect of learning algorithm:** It has been suggested that the learning algorithms have a minor effect in the classification accuracy of pre-miRNA [2]. To investigate this statement, we compared the classification accuracy of classification models obtained in the proposal 1, which induced using four algorithms representing different learning biases and FSi , for $i = 1, \dots, 7$.
3. **Prediction of G+C-rich sequences:** G+C content is an important feature associated with the folding of hairpin-like RNA sequences. Because G+C-rich sequences have more alternative high-energy stable binding-pairs, the prediction of the corresponding secondary structure is more complex. We investigated the sensitivity and specificity of the feature sets in the proposal 1 throughout the 12.5%-G+C content quantiles distribution.
4. **Generalization of feature sets across species:** It is a fact that the hairpin structure varies largely within and between species. To investigate the generalization of the discriminant power of the feature sets FSi , for $i = 1, \dots, 7$ in other species, the estimated accuracies of classifiers induced using those feature sets, three different learning algorithms and training instances of 45 species were obtained.
5. **Cross-species models:** Due to the low availability or lack of positive examples for many species, it is important to investigate the worth of cross-species models. To investigate a classification model induced with positive and negative examples from a species i can predict with non-significantly different accuracies the class of instances from another species j , with $i, j = 1 \dots 45$, the classifiers obtained under the proposal 4 was tested in the test sets of all species.

6. **Alternative feature sets:** Computation of features is the most costly step in computational analysis of putative pre-miRNA. We investigated alternative feature arrangements taking into consideration their computational cost and their discriminant power.
7. **Ensembles:** Another way to reduce the cost of the computational analysis was tried using ensembles of computationally less expensive feature sets. Since majority and weighted votes are the most basic approaches to construct ensembles, the ensemble approaches investigated in this work were:
 - (a) majority vote of classifiers produced by less expensive feature sets and one specific learning algorithm;
 - (b) weighted vote of classifiers produced by less expensive feature sets and one specific learning algorithm. The weights were the accuracies obtained by each classifier;
 - (c) majority vote of classifiers produced by less expensive feature sets and three learning algorithms;
 - (d) weighted vote of classifiers produced by less expensive feature sets and three learning algorithms. The weights were the accuracies obtained by each classifier.

1.9 Hypotheses

We investigated three main hypotheses aiming to make inferences about the impact of the main factors involved in the induction of classification models for pre-miRNA prediction, namely: the feature set describing positive and negative sequences, the learning algorithm and the species whose sequences are taken to compose training sets. The first and second hypotheses were investigated by comparing the average estimated accuracies of classifiers induced using the feature sets $FS_1, \dots, FS_7$ ($H1_0$) and two learning algorithms. These investigations are justified by the impossibility to infer the contribution of different feature sets and learning algorithms in the estimated accuracies of existing computational tools for pre-miRNA classification. Since those feature sets vary largely in composition and computational cost, it is important to compare their discriminant power. Moreover, as pointed in [2], learning algorithms seem to have a minor effect in the predictive accuracy of pre-miRNA classifiers. The third hypothesis is justified by the need of training examples from a species to induce classification models to predict putative pre-miRNAs from other species. These three hypotheses are symbolically given below:

1. There is no difference between the predictive performance of the classifiers induced with $FS_1, \dots, FS_7$, under the same learning algorithm, training and test sets.
 $H1_0: \mu_{FS_i} = \mu_{FS_j}$, for $i, j = 1, \dots, 7$ and $i \neq j$ and μ is the average performance.
2. Given a feature set, there is no difference between the predictive performance of the classifiers induced by algorithms A_1, A_2, A_3 , under the same training and test sets.

H2₀: $\mu_{A_i} = \mu_{A_j}$, for $i, j = 1, 2, 3$ and $i \neq j$ and μ is the average performance.

3. Models induced with instances from a species i can predict with non-significantly different accuracies instances from a species j , for $i, j = 1, \dots, 45$ and $i \neq j$.

H3₀: $\mu_{spe_i} = \mu_{spe_j}$, for $i, j = 1, \dots, 45$ and $i \neq j$ and μ is the average performance.

1.10 Outline

In Chapter 2 we present the methodological background and the experimental designs applied in the computational experiments and in the analyzes carried out in this work. The results of our investigation are presented in three chapters. In Chapter 3, the main aspects affecting the predictive performance of human pre-miRNA classifiers are identified (proposals 1–3). As a result of the experiences on the predictive power of RNA features used for pre-miRNA recognition, we obtained a lower-dimensional feature set, which achieved average sensitivity of 90% and average specificity of 95%. These estimates are within 0.1% of the maximal values obtained with any feature set, while are 34 times faster to compute than the least expensive feature set which produced the highest average accuracy. The results from the proposals 4 and 5 are presented in Chapter 4. Essentially, we discuss if the prediction of pre-miRNAs from different species constitute different learning problems. Surprisingly, we show that the pre-miRNA prediction of even phylogenetically close species may correspond to different learning problems. Finally, the results from the proposals 6 and 7 are discussed in Chapter 5, where we provide evidences that ensembles of classifiers induced with computationally inexpensive feature sets can both increase the classification performance and reduce the computational costs of feature extraction. In Chapter 6, we summarize our main observations from the results discussed in chapters 3–5, where we also point out some limitations of our results, raising questions that could be an issue for further investigation and mention prospective research on the topic of miRNA discovery.

Conclusion

The analysis of genomic sequences to detect loci likely to contain miRNAs using current *ab initio* methods has been limited by two main problems: they can produce high false detection rates and/or they require the computation of features computationally costly. We considered computationally costly feature sets those containing stability measures, which are features computed from usually 1,000 shuffled versions of each putative pre-miRNA sequences. Since the expected number of putative miRNA loci to be analyzed is usually in the order of millions, the analysis of putative microRNAs using methods leading to high false detection rates and/or using computationally expensive feature sets would be wasteful or even unfeasible for large genomes.

To seek feature sets that closely represent the true space of real and pseudo pre-miRNAs, the discriminant power of 1,385 RNA features proposed in the literature was investigated individually and in different feature sets. Particularly, the specificity of 85 out of those 1,385 features were investigated in 45 species, regarding their relevance in the classification of putative pre-miRNAs from those species. The results of these investigations, presented and discussed in chapters 3, 4 and 5, led to the proposition of alternative feature sets, named SELECT (see Chapter 3), Hyb₃₇, Hyb_{S7}, Hyb₁₇ and Ss1 (see Chapter 5). The main characteristic of these feature sets is the absence of stability measures. The proposed features sets are not only computationally less costly than those feature sets from the literature containing stability measures, but they produced classifiers whose predictive accuracies were equal to or greater than the predictive accuracies produced by the feature sets from the literature containing stability measures. These results suggest that the stability measures and other features might be highly correlated, although we have not computed the correlations.

Regarding to the hypotheses $H1_0$ and $H2_0$ stated in Chapter 1, our results corroborated with their rejection. As a consequence, we provided statistical evidences to conclude that the average accuracies of predictive models obtained with different feature sets, but the same learning algorithms, are not all equal. Likewise, we also concluded that the average accuracies of predictive models obtained with different learning algorithm, but with the same feature sets, are not all equal. Nevertheless, the differences in accuracies due to the learning algorithm are significantly lower than the differences in accuracies due to the feature sets. It is important to stress that these hypotheses were evaluated using large training data sets (1,742 instances) computed from human sequences. Therefore, the differences in accuracies due to algorithms may be larger when smaller training sets are used as it was observed in Figure 3.1 and also in Table C.1, where the accuracies were estimated using smaller training data sets (120 instances) from different species.

The hypothesis $H3_0$ was tested using the results of classifiers induced with training data computed from each of the 45 species, in predicting the classes of instances from other species. These results produced a 45×45 matrix, from where the t -test was applied to evaluate if the accuracies of a model species i in predicting the classes of instances from the species i and j , $i \neq j$, were not significantly different. As stated in Chapter 1, the purpose of investigating this hypothesis was to identify the cases where cross-species predictive models would be viable. The rejection of $H3_0$ was associated with the model species whose accuracy was low, compared to the accuracies from other model species. As it was mentioned in Chapter 4, the results of these tests added evidences that the classes of putative pre-miRNAs from certain species are computationally easier to predict than the classes of instances from other species. Moreover, these results also showed that the choice of the training instances may be better oriented by the complexity of the available instances than by the phylogenetic proximity among the species.

Finally, this work also presented preliminary results of investigations on ensemble models for pre-miRNA prediction. The main outcome from the results of these investigations was the indication that ensembles of classifiers induced with computationally less expensive feature sets achieved accuracies in the highest accuracy cluster for a larger number of species than simple classifiers induced with feature sets containing the computationally expensive stability measures. As a consequence, ensembles were pointed as an approach to develop pre-miRNA predictive models of higher accuracies, while keeping the computation of the feature sets within the computational time of the less costly feature sets.

6.1 Contributions

The main contributions from this work are:

1. The proposal of standardized methodological frameworks, described in Chapter 2, Section 2.4 allowing the isolation of controllable variation sources in the comparison of the induced models;
2. The estimates of accuracies and of computational time of existing feature sets, in Chapter 3;
3. The proposal of less costly feature sets likely to provide the same accuracies of computationally costly feature sets from the literature;
4. The experimental results were obtained for the largest number of species already investigated in the literature;
5. The investigation of cross species models, detailed in Chapter 4;
6. The detailed statistical analysis of the experimental results allowing better interpretation and inferences;
7. The analyses showing the variable learning complexity of instances from different species, which can be more relevant for classification than the phylogenetic proximity;
8. The proposal of ensemble-based approaches to increase the predictive performance and to reduce the computational time necessary to compute the feature sets.

6.2 Limitations

To isolate the controllable sources of variation in the computational experiments investigating the effect of the species, the training sets size was limited to 60 instances from each class, which corresponded to $2/3$ of the smallest positive set. Therefore, the accuracy estimates presented in this work are subject to high standard deviations, than those presented in Table 3.2. Moreover, the representativeness of positive examples may vary among species. A possible consequence would be less generalizable models, which would achieve low accuracies in the prediction of test sets, even from the same species. Nevertheless, we found no indication of such relationships, since the classes of instances from test sets from the species whose models achieved low accuracies were systematically predicted with low accuracy by other models species.

6.3 Prospective work

As our results showed that the learning complexity of pre-miRNAs vary throughout species and that ensembles are a promising approach to increase the predictive accuracy of classification

models for a large number of species - a natural continuation of this work is to develop a classification tool that take advantage of these two results. Particularly, the induction of ensemble members with examples from more complex species described by different feature spaces, but still computationally less costly, may produce predictive models more likely to achieve higher accuracies in the classification of instances from any species. Along with the development of accurate pre-miRNA classification tools, new methods for the automatic excision of putative pre-miRNAs from different species are necessary to advance the discovery of miRNAs using *ab initio* tools.

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